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A COMPARATIVE STUDY OF THE METABOLISM OF CERTAIN AMINO ACIDS, WITH SPECIAL REFERENCE TO RESPIRATORY EXCHANGE AND HEAT PRODUCTION [1]

MAX KRISS

Institute of Animal Nutrition, The Pennsylvania State College, State College

ONE FIGURE

(Received for publication October 10, 1938)

In connection with an investigation of the specific dynamic effects of glycine, alanine and glutamic acid it was necessary to determine the metabolizability of the amino acids, and the factors for computing the respiratory exchange and the heat production. Kriss and Voris ('37) have determined the respiration and energy factors for heart muscle protein, casein and gelatin, and have found them to differ with the type of protein. These differences are presumably related to the amino acid composition of the proteins.

Lusk ('12–'13, '15) calculated the respiration and energy factors for a number of amino acids, including glycine, alanine and glutamic acid, on the basis of theoretical considerations, by accounting for the values of urea formed from them in metabolism. These physiological values (liters of O_2 and CO_2, and calories per gram of nitrogen) are greatly different for the different amino acids. The values for alanine are approximately twice as great as the corresponding values for glycine. The values for glutamic acid are approximately three times as great as those for glycine, while the values for tyrosine are approximately six times the corresponding values for

[1] Authorized for publication on October 8, 1938, as paper no. 855 in the journal series of the Pennsylvania Agricultural Experiment Station.

glycine. In general, there is a close inverse correlation between the magnitude of these factors and the nitrogen content of the amino acids. No experimentally determined factors for computing the heat production and the respiratory exchange in the metabolism of amino acids seem to be available.

The experiments recorded in the present paper are concerned with the determination of the metabolizability of glycine, alanine and glutamic acid, and with the derivation of the respective respiration and energy factors from balances of carbon, nitrogen and energy.

EXPERIMENTAL

The subjects of these experiments were sixteen male albino rats, approaching maturity. The rats weighed somewhat more than 200 gm. each when they were placed on restricted food intake. The first experimental dietary treatment was a basal ration of 8 gm. per day, this ration consisting of 93.7% of calf meal (described by Forbes, Kriss and Miller, '34) and 6.3% of butterfat. During the first few days on this diet the animals lost some weight, but they soon came into equilibrium with the diet, which was adequate for the maintenance of approximate equilibrium of nitrogen and energy. All animals were kept on this diet for a period of approximately 2 weeks before any of the balance experiments were begun.

This preliminary feeding period was followed by a 7-day period on the basal ration during which the feces and urine were collected from each rat.

Following this treatment the animals, divided into three groups, were offered, in addition to the basal ration, supplementary portions of different amino acids [2] in quantities supplying 7500 calories of gross energy per day. Six rats received 2.06 gm. of d-glutamic acid each per day. One animal of this group soon showed hematuria and was therefore discarded. Six other rats received 1.75 gm. of dl-alanine each per day. These two groups received the supplemented diet for a period of 8 days. Feces and urine were collected during

[2] Purchased from Eastman Kodak Co., Rochester, N. Y.

the last 5 days. A third group of five rats was offered glycine as a supplement to the basal ration. Only one animal of this group (rat 11) consumed the full amount (2.4 gm. per day) calculated to supply 7500 calories, for a period of 8 days. Feces and urine were also collected from this rat during the last 5 days. The other animals of this group, after receiving the full amount of glycine for a few days, ceased to consume this amount completely. Thereupon the quantity of glycine was reduced to 1.8 gm. (equal to 75% of the original amount) and was fed for 8 days. During the last 5 days of this period feces and urine were collected.

After the completion of the excreta collection period for the animal (rat 11) which consumed 2.4 gm. of glycine per day, the quantity of glycine fed this rat was also diminished to 1.8 gm. for a period of 8 days, during the last 5 of which excreta were collected.

The urine of each individual rat was analyzed for nitrogen and carbon. Energy was determined in the urine on a composite sample. The feces were composited for each group before being subjected to similar analyses. The basal rations and the amino acids used as supplements were also analyzed for nitrogen, carbon and energy. The excretory products representing the amino acid supplements were determined as the differences between the residues from the basal ration and the supplemented diets.

RESULTS

Table 1 sets forth the analyses of the basal ration and of the amino acid supplements. The analyses indicate a high degree of purity of the amino acids. In table 2 are presented the results of the urinary analysis. These data are characterized by a high degree of consistency. It is of especial interest to note that the carbon: nitrogen ratios representing the urinary constituents of glutamic acid and of glycine are only slightly higher than the carbon: nitrogen ratio (0.428) in urea, while the carbon: nitrogen ratios representing the urinary constituents derived from alanine are more than

twice as high as the carbon : nitrogen ratio characteristic of urea. Similarly, the energy : nitrogen ratios representing the urinary constituents of glutamic acid and of glycine are only slightly higher than the energy : nitrogen ratio (5.447 calories per milligram of nitrogen) found in urea, while the energy : nitrogen ratio in the urinary excretions from alanine is more than twice as high as the energy : nitrogen ratio of urea.

These ratios of carbon to nitrogen and of energy to nitrogen indicate that the urinary excretions from the amino acids contain substances other than urea, with higher C : N and energy : nitrogen ratios than those characterizing urea.

TABLE 1

Analysis of basal ration and of amino acid supplements

MATERIAL	NITROGEN	CARBON	ENERGY
	%	%	*Calories per gram*
Basal ration	3.27	43.49	4.481
d-glutamic acid	9.30	40.87	3.651
Basal ration	3.27	43.49	4.481
Glycine	18.47	32.25	3.098
Basal ration	3.38	44.05	4.529
dl-alanine	15.50	40.50	4.354

Lusk ('12–'13) made the observation that, in feeding glutamic acid, glycine and alanine to a dog, fractions of unoxidized amino acid were excreted in the urine, these fractions being larger after the ingestion of alanine than after the ingestion of either glycine or glutamic acid. The C : N ratios, as well as the energy : N ratios found in the urine of our rats, are entirely in harmony with these observations of Lusk and may have a similar significance.

In order to arrive at approximate estimates of the quantities of unmetabolized amino acids which passed into the urine, it was first assumed that the differences between the urinary C : N ratios obtained for the particular amino acid and the C : N ratio existing in urea were due entirely to the excretion,

TABLE 2

Urinary analysis

AMINO ACID SUPPLEMENT AND RAT NO.	NITROGEN PER DAY		CARBON PER DAY		ENERGY PER DAY		DIFFERENCES DUE TO AMINO ACIDS				
	Basal ration	Amino acid supplemented ration	Basal ration	Amino acid supplemented ration	Basal ration	Amino acid supplemented ration	Nitrogen	Carbon	Energy	C : N ratio	Energy : N ratio
	mg.	*mg.*	*mg.*	*mg.*	*cals.*	*cals.*	*mg.*	*mg.*	*cals.*		
2.06 gm. glutamic acid											
1	167	323	126	197			156	71		0.455	
2	180	337	130	205			157	75		0.478	
3	173	298	126	223			125	97		0.776	
4	174	308	125	200			134	75		0.560	
5	174	320	128	199			146	71		0.486	
Average	174	317	127	205	1381	2254	143	78	873	0.545	6.105
1.8 gm. glycine											
11	173	473	131	280			300	149		0.497	
12	167	469	129	276			302	147		0.487	
13	181	493	136	295			312	159		0.510	
14	154	453	125	284			299	159		0.532	
15	167	451	121	270			284	149		0.525	
Average	168	468	128	281	1425	3235	300	153	1810	0.510	6.033
2.4 gm. glycine											
11	173	589	131	349	1425	4062	416	218	2637	0.524	6.339
1.74 gm. alanine											
21	159	408	117	365			249	248		0.996	
22	160	392	119	371			232	252		1.086	
23	164	386	125	361			222	236		1.063	
24	163	386	121	348			223	227		1.018	
25	153	374	111	370			221	259		1.172	
26	170	384	124	343			214	219		1.023	
Average	162	388	120	360	1303	3903	226	240	2600	1.062	11.504

in the urine, of unmetabolized amino acid; and from these differences the quantities of unoxidized amino acids were computed. Then, in a similar manner, the quantities of unchanged amino acid excreted in the urine were computed on

TABLE 3
Metabolizability of amino acids

	GLUTAMIC ACID 2.06 GM.	GLYCINE		ALANINE
		2.4 gm.	1.8 gm.	1.74 gm.
Income				
Energy, calories	7.521	7.435	5.576	7.576
Carbon, grams	0.842	0.774	0.581	0.705
Nitrogen, grams	0.192	0.443	0.332	0.270
Outgo				
Feces				
Energy, calories	0.569	.....	.....	.....
Carbon, grams	0.056	.....	.....	.....
Nitrogen, grams	0.010	.....	.....	.....
Urine				
Energy, calories	0.873	2.637	1.810	2.600
Carbon, grams	0.078	0.218	0.153	0.240
Total nitrogen, grams	0.143	0.416	0.300	0.226
Unmetabolized amino acid N, grams [1]	0.004	0.031	0.019	0.061
Unmetabolized amino acid N, grams [2]	0.003	0.033	0.016	0.062
Metabolized amino acid N, grams [1]	0.139	0.385	0.281	0.159
Metabolized amino acid N, grams [2]	0.138	0.383	0.284	0.164
Metabolized amino acid N, per cent [1]	97.20	92.55	93.67	70.35
Metabolized amino acid N, per cent [2]	96.50	92.07	94.67	72.57
Metabolizable				
Energy, calories	6.079	4.798	3.766	4.976
Carbon, grams	0.708	0.556	0.428	0.465
Energy, per cent	80.83	64.53	67.54	65.68
Carbon, per cent	84.09	71.83	73.67	65.96

[1] Calculated on the basis of the C: N ratios.
[2] Calculated on the basis of calories: N ratios.

the basis of the differences between the observed urinary energy: nitrogen ratios and the energy: nitrogen ratio of urea. The values obtained for unmetabolized amino acid nitrogen in the urine by the two methods (table 3) are practically identical, and, since they are based on different analytical

procedures, their agreement can be considered as serving to substantiate the essential correctness of the assumption involved.

It should be stated that the foregoing calculations do not preclude the possibility of the presence in the urine, besides urea and unoxidized amino acid, of other products of incomplete oxidation of the amino acid, with a combined C:N ratio and a combined energy:nitrogen ratio corresponding to the ratios found in the unchanged amino acid. This and any other theoretical considerations, however, which might affect to some extent the partitioning of the urinary nitrogen, would be without significant effect on the final respiration and energy values on which the graphs in figure 1 are based, inasmuch as those values are expressed in relation to the total urinary nitrogen.

The quantities of nitrogen in the urine representing the amino acids metabolized were calculated as the differences between the total urinary nitrogen and the unmetabolized amino acid nitrogen. Calculated from the C:N ratios, the average quantities of nitrogen in the urine derived from the amino acids metabolized constitute 97.20% of the total urinary nitrogen for glutamic acid, 92.55% and 93.67%, respectively, for the higher and lower levels of glycine, and 70.35% for alanine. The same values calculated from the calories:N ratios are 96.50% for the glutamic acid, 92.07% and 94.67%, for the two levels of glycine, and 72.57% for alanine. These values are in substantial agreement with those found by Lusk ('12–'13), in the dog, on the basis of determinations of urea plus ammonia nitrogen, and total nitrogen, in the urine. Lusk's estimate for the deaminized portion of alanine was 70%, and for glycine 96.06%, and 94.46%, in two experiments.

In all of the foregoing calculations it was tacitly assumed that the protein metabolism was not altered by the addition of the amino acid supplements to the basal ration. Direct proof of the correctness of this assumption is not feasible, but it is substantiated indirectly by the consistent character of

the results. An alternative assumption, that the contribution to the protein metabolism by the basal ration in the supermaintenance periods is represented by the difference between the total urinary nitrogen of the supermaintenance period and the total amino acid nitrogen ingested or absorbed, leads to the calculation of C : N ratios in the urinary residues from the amino acids, which are in all cases considerably higher than the C : N ratios found in the urine from the basal ration. This would not be consistent with the large quantities of urea known to be formed from the amino acids and excreted in the urine, especially in the case of glycine feeding. The second assumption must, therefore, be discarded in favor of the first.

The results of the fecal analyses were such as to indicate that glycine and alanine were completely absorbed from the intestinal tract, and that small quantities of glutamic acid were eliminated in the feces. The differences between the daily fecal outgo from the ration supplemented with glutamic acid and the basal ration were 10 mg. nitrogen, 56 mg. carbon, and 569 calories. These values were all positive and their relations to each other are not much different from those existing in the amino acid. With glycine and alanine the differences representing fecal elimination were negligibly small. It is possible that elimination of small quantities of glutamic acid through the intestine occurred because of a relatively slow rate of absorption of this amino acid (Wilson and Lewis, '29).

In table 3 are presented the balances of nitrogen, carbon and energy representing the different amino acids. The metabolizable energy or carbon of the amino acids is computed as the difference between the total energy or carbon of the substance ingested and the corresponding energy or carbon, of the excreta. In this computation the energy of the urine was not corrected to represent nitrogen equilibrium.

Of the total nitrogen ingested as amino acids the following percentages were eliminated in the urine: from glutamic acid, 74.48%, from glycine, 93.91% and 90.36%, for the higher and lower levels, respectively, and from alanine, 83.70%.

Of the energy ingested as glutamic acid 80.83% was metabolized; the energy of glycine was metabolized to the extent of

TABLE 4

Determination of factors for computing the respiratory exchange and the heat production in the metabolism of glutamic acid, glycine and alanine

	GLUTAMIC ACID 2.06 GM.	GLYCINE		ALANINE 1.74 GM.
		2.4 gm.	1.8 gm.	
Intake, computed per 100 gm. of pure amino acid				
Energy, calories	365.10	309.80	309.80	435.40
Nitrogen, grams	9.52	18.66	18.66	15.73
Carbon, grams	40.79	31.98	31.98	40.42
Hydrogen, grams	6.17	6.72	6.72	7.92
Oxygen, grams	43.51	42.64	42.64	35.93
Outgo				
Feces				
Energy, calories	28.21			
Nitrogen, grams	0.50			
Carbon, grams	2.78			
Hydrogen, grams	0.32			
Oxygen, grams	2.29			
Urine				
Energy, calories	55.07	118.28	112.58	180.96
Total nitrogen, grams	9.02	18.66	18.66	15.73
Unmetabolized amino acid N, grams	0.25	1.44	1.12	4.52
Metabolized (urea) N, grams	8.77	17.22	17.54	11.21
Carbon, grams	4.92	9.78	9.52	16.70
Hydrogen, grams	1.41	2.98	2.91	3.88
Oxygen, grams	6.15	13.13	12.58	16.74
Metabolized				
Energy, calories	281.82	191.52	197.22	254.44
Carbon, grams	33.09	22.20	22.46	23.72
Hydrogen, grams	4.44	3.74	3.81	4.06
Oxygen, grams	35.07	29.51	30.06	19.19
Intramolecular H_2O				
Hydrogen, grams	4.38	3.69	3.76	2.40
Oxygen, grams	35.07	29.51	30.06	19.19
Carbon oxidized to CO_2, grams	33.09	22.20	22.46	23.72
Hydrogen oxidized to H_2O, grams	0.06	0.05	0.05	1.66
CO_2 produced, grams	121.33	81.40	82.35	86.97
CO_2 produced, liters	61.81	41.47	41.95	44.30
O_2 required to oxidize C, grams	88.24	59.20	59.89	63.25
O_2 required to oxidize H, grams	0.48	0.40	0.40	13.28
Total O_2 required, grams	88.72	59.60	60.29	76.53
Total O_2 required, liters	62.09	41.71	42.19	53.56
Respiratory quotient	0.995	0.994	0.994	0.827
Liters O_2 required per gram urinary N	6.88	2.24	2.26	3.40
Liters CO_2 produced	6.85	2.22	2.25	2.82
Calories metabolized per gram urinary N	31.24	10.26	10.57	16.18
Calories per liter O_2	4.491	4.592	4.675	4.751
Liters O_2 required per gram N of amino acid metabolized	7.08	2.42	2.41	4.78
Liters CO_2 produced per gram N of amino acid metabolized	7.05	2.41	2.39	3.95
Calories metabolized per gram N of amino acid metabolized	32.13	11.12	11.24	22.70

64.53% (for the higher level) and 67.54% (for the lower level). The metabolizable energy of alanine was 65.68% of the total.

The percentages of carbon metabolized were 84.09 for glutamic acid, 71.83 and 73.67 for glycine, and 65.96 for alanine.

The relatively low metabolizability of the energy of glycine as compared with glutamic acid is apparently due to the greater quantity of urea formed from the former, while the relatively low metabolizability of the energy of alanine is apparently due, to some extent, to the relatively large quantity of unchanged amino acid eliminated in the urine.

The derivation of the respiration and energy factors for the different amino acids is shown in table 3. The computations are on the basis of pure amino acids.

The values for nitrogen, carbon and energy of feces and urine, per 100 gm. of amino acid, were computed on the basis of the data given in table 2, in relation to the nitrogen content of the amino acids. The values for hydrogen and oxygen in the feces from glutamic acid were computed from the nitrogen on the assumption that the fecal excretion was in the form of the amino acid. The values for hydrogen and oxygen in the urine were computed on the basis of the calculated values of urea and of unmetabolized amino acid present. All other calculations for the derivation of the respiration and energy factors are self-explanatory.

The final results obtained with each of the amino acids tested (the values for glycine being given as the average for the two levels) are summarized as follows:

	Glutamic acid	*Glycine*	*Alanine*
Respiratory quotient	0.995	0.994	0.827
Calories metabolized per liters O_2	4.491	4.634	4.751
Liters O_2 required per gram urinary N	6.88	2.25	3.40
Liters CO_2 produced per gram urinary N	6.85	2.24	2.82
Calories metabolized per gram urinary N	31.24	10.42	16.18
Liters O_2 required per gram N of amino acid metabolized	7.08	2.42	4.78
Liters CO_2 produced per gram N of amino acid metabolized	7.05	2.40	3.95
Calories metabolized per gram N of amino acid metabolized	32.13	11.18	22.70

The values for O_2, CO_2 and calories per gram of total urinary nitrogen do not differ very much from the corresponding values expressed per gram of nitrogen of amino acid metabolized, in the case of glutamic acid or of glycine. In the case of alanine, however, the factors expressed per gram of nitrogen of the amino acid metabolized are considerably higher than those expressed per gram of total urinary nitrogen. This is accounted for by the fact, previously shown (table 3), that relatively small quantities of unmetabolized glutamic acid or glycine appeared in the urine, while a considerable porportion of the ingested alanine was eliminated in the urine in unchanged form. The respiration and energy factors for all three amino acids under consideration, expressed per gram of nitrogen of amino acid metabolized, agree very closely with the corresponding theoretical values calculated by Lusk ('12–'13, '15). The factors per gram of total urinary nitrogen, however, correctly apply to the experimental conditions.

A close examination of the respiration and energy factors which have been determined for the different amino acids, in the present study, and of the factors for proteins reported previously by Kriss and Voris ('37), in relation to the elementary composition of these substances, reveals that while there is, in general, a fairly close correlation between these factors and the nitrogen content of the substances, a still closer correlation exists between these factors and the ratios of carbon to nitrogen in the materials. The latter relationship is shown graphically in figure 1.

The graphs show consistently higher values for O_2, CO_2 and calories per gram of total urinary nitrogen, with the proteins than with the single amino acids, thus indicating that the metabolism (oxidation) was more complete with the former than with the latter. The graphs representing the amino acids do not and probably would not apply to sulphur-containing amino acids.

While the data on which figure 1 is based are few, they clearly indicate that when more experimental data of this sort become available it will probably be possible to derive

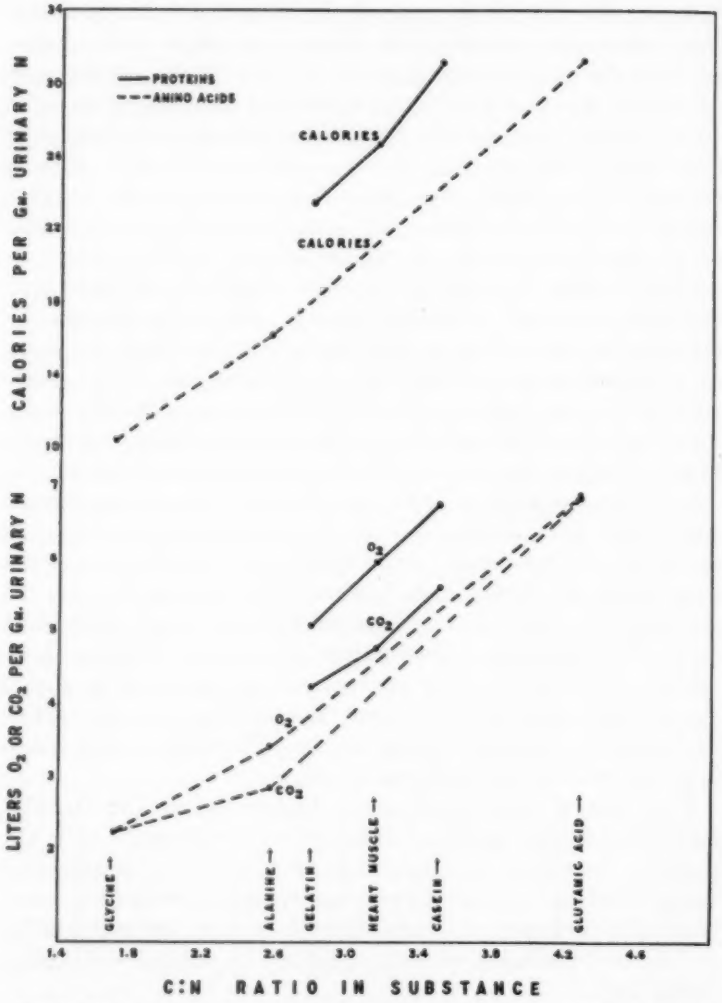

Fig. 1 Relation of respiration and energy factors for amino acids and proteins to the ratios of carbon to nitrogen in these substances.

a general formula, for practical use in indirect calorimetry, for the calculation of respiration and energy factors for any protein, and possibly for any amino acid. Tentatively, there seems to be good reason to suggest that the factors for proteins the C : N ratios of which lie within the range of 2.79 (gelatin) and 3.50 (casein) can be calculated from the C : N ratios in the substance, on the basis of the data for proteins represented in figure 1, by interpolation.

SUMMARY

Glutamic acid fed to rats as a supplement to a mixed maintenance ration, in a quantity supplying 7.5 kilogram-calories of gross energy per day, was absorbed to the extent of 95%, while glycine fed as a supplement to the same basal ration, in quantities supplying 7.4 and 5.6 kilogram-calories, and alanine fed in a quantity supplying 7.5 kilogram-calories per day, were completely absorbed. From the ratios of carbon to nitrogen and of energy to nitrogen in the urine, it was calculated that approximately 3% of glutamic acid, 7% of glycine and 29% of alanine, respectively, were excreted in the urine in the form of the unoxidized amino acids, the remaining portions of the urinary nitrogen, derived from the amino acids, representing the amino acids metabolized. The metabolizable energy of glutamic acid was found to be 80.8% of its gross energy, while the metabolizability of the energy of alanine was only 65.68%, and that of glycine 64.53%, at the higher level, and 67.54% at the lower level of feeding.

Factors were determined for computing the respiratory exchange and the heat production in the metabolism of each of the three amino acids. These factors were found to be closely correlated with the ratios of carbon to nitrogen in the different amino acids. A similar correlation was found to exist between the respiration and energy factors for some proteins, namely, casein, gelatin and heart muscle protein, and the ratios of carbon to nitrogen in these substances. A graphic representation of these relationships indicates that the proteins were more completely metabolized than were the

single amino acids. A tentative method has been suggested for making practical use of these relationships in indirect calorimetry.

The author is indebted to Mr. Virgil V. Herring for carrying out the determinations of carbon in connection with this investigation.

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THE LEVEL OF INORGANIC PHOSPHORUS IN THE BLOOD OF DAIRY CATTLE [1]

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(Received for publication October 1, 1938)

In connection with a study of phosphorus deficiency in the cattle of Pennsylvania it was necessary to establish the normal content of inorganic phosphorus in the whole blood. A series of determinations of this value were made, therefore, with well-fed cattle as the experimental subjects.

As pointed out by Hamersma ('37), diverse results have been obtained from determinations of inorganic blood phosphorus in different laboratories in accord with the method employed—the estimations having usually been made on the serum or the plasma.

Under field conditions such that the blood cannot be chilled or analyzed at once, it is more desirable to use whole blood for the determination, for reasons discussed by Stare and Elvehjem ('32). These authors, and Van Landingham, Henderson and Bowling ('35), have determined the inorganic phosphorus of the whole blood of immature dairy cattle, and both these groups of workers used the method of Fiske and Subbarow.

Recently Van Landingham and co-workers ('38) presented values for the inorganic phosphorus of the blood of successive periods of lactation, as follows:

1st lactation 4.95 mg. per 100 ml.
2nd lactation 4.81 mg. per 100 ml.
3rd lactation 4.35 mg. per 100 ml.
4th lactation 4.33 mg. per 100 ml.

[1] Authorized for publication on September 9, 1938, as paper no. 850 in the journal series of the Pennsylvania Agricultural Experiment Station.

In addition to the recognized effects of age and of feed on the inorganic phosphorus level in blood, the stage of lactation (Van Landingham, Henderson and Bowling, '38), and the amount of milk produced (Van Landingham, Henderson and Bowling, '38; Becker, Neal and Shealy, '33; Eckles, Gullickson and Palmer, '32; Groenewald, '35) are said also to exert appreciable effects.

Becker, Neal and Shealy ('33) give values for nursing cows of 2.87 mg., for dry cows of 3.80 mg. and for steers and heifers of 4.91 mg. per 100 cc., all representing cattle in phosphorus deficient areas.

The specific purposes of the present study were to establish standard, normal values for the inorganic phosphorus of the whole blood of dairy cattle, and to study the variations in these values resulting from differences in conditions as to breed, age, milk production, reproductive status and other factors on which information could be obtained.

EXPERIMENTAL

Inorganic phosphorus was determined on blood samples from over 200 cows and heifers by the method of Youngburg and Youngburg ('30). Immediately after drawing the sample, the quantity of 2 cc. was pipetted into 8 cc. of 10% trichloracetic acid. The samples were filtered as soon as possible, being kept cold in the meantime, though preliminary studies indicated that these precautions were probably unnecessary.

The animals of two separate herds at the college, one of which is maintained primarily for instructional and the other for research purposes, were used. No attempt was made to include every animal, and, in any case in which the drawing of the blood caused excitement of the animal, the sample was rejected.

The research herd, referred to as herd 1, was receiving a standard, balanced, concentrate mixture, and timothy hay, and either corn or alfalfa silage. The instructional herd, designated herd 2, received a balanced ration of oats, corn, peanut bran, soy bean meal, brewers grains, wheat bran, molasses and salt, and mixed or alfalfa hay and corn silage.

All samples were taken during the spring, when the animals had been away from pasture for 4 months or longer.

The data were grouped in several different ways. In some cases, in which significant differences between the average values for groups which were studied were not found, it is possible that a study of larger populations might uncover definite differences.

In herd 1, the values for the animals on corn silage and for those on alfalfa silage were not significantly different; neither were there important differences in accord with the breed and the age of adult cattle, though in the case of heifers the age groups did differ as to blood phosphorus, and significant differences were found between the milking and the dry cows. Two cows that were being dried off, and were giving less than 10 pounds of milk daily, were included in the dry-cow group. All other cows were giving 25 pounds or more of milk daily. Within the small group of twenty-six milking cows no definite correlations between milk production and inorganic phosphorus in the blood was found.

In herd 2, only five of the cows tested were dry, and their inorganic blood phosphorus values were similar to the average for those which were giving milk. There was no difference between the averages for cows giving 10 to 24 and those giving 25 to 40 pounds of milk, and a small difference between those giving 25 to 40 pounds and others giving over 40 pounds of milk daily was not statistically significant. In this herd, also, no differences were found between breeds nor between the 2- to 3-year-old and the 4- to 5-year-old milking cows, but a significant difference was found between averages for 4- to 6-year-old cows and cows which were more than 6 years old.

In both herds cows which had calved within 2 weeks were excluded, since their inorganic blood phosphorus values were quite variable, and in most cases were abnormally high. Only a few males were examined. They were all young, and the values obtained could not be distinguished from those for heifers of the same age.

The averages for the groups of heifers from the two herds were almost identical. They were therefore combined, and were then divided into groups differing as to age by approximately 6 months. The relation of age to level of inorganic phosphorus in the blood of immature cattle is clearly shown in tables 1 and 2. The range of individual values also descended uniformally with advance in age, except for the low value of 4.1 mg. per 100 ml. in the 14- to 19-month-old group. The next lowest value in this group was 4.8 mg., which is in line with the lower limit of the other age groups. If the average values of the five age groups are plotted against the ages of these groups the result is a nearly straight line representing diminishing inorganic blood phosphorus with increasing age. In herd 2 there was also a significant difference between the inorganic blood phosphorus of cows 4 to 6 years of age, and cows 6 years of age or older.

The average values for inorganic blood phosphorus of the milking cows in herd 2, and of the dry cows in both herds were virtually the same, 4.33 mg. and 4.35 mg., respectively, per 100 ml. of whole blood. The value 4.11 mg. for the milking cows of herd 1 is significantly lower than the above values, while the heifers and dry cows of this herd gave values in harmony with those of herd 2. This inconclusive evidence indicates that the ration for the cows in herd 1 was not sufficiently high in phosphorus to enable cows in full lactation to maintain a normal level of blood phosphorus.

The results suggest that milk production has little or no influence on the level of inorganic phosphorus in the blood when the animals are under optimum conditions of feed and management. This does not preclude the probability that milk production has an effect on the blood phosphorus values of animals on restricted feed intake, or on rations of low phosphorus content.

In field studies carried out in connection with this work, very few low values for the inorganic blood phosphorus of dry cows were found, even with low phosphorus intake. A similar observation was also made by Becker and associates

TABLE 1

Average inorganic phosphorus contents of the whole blood of dairy cattle. Milligrams per 100 ml.

GROUP	DESCRIPTION	NUMBER	AGE	DAILY MILK	MEAN, INORGANIC P	PROBABLE ERROR OF MEAN	STANDARD DEVIATION	RANGE, INORGANIC P
			months	*pounds*				
	Herd 1							
1	Milking cows	26	57	44	4.11	0.06	0.48	3.3–5.3
2	Dry cows	12	76	..	4.39	0.07	0.36	3.7–4.9
	Herd 2							
3	Cows in milk, 2 to 3 years old	25	40	30	4.42	0.05	0.38	3.7–5.3
4	Cows in milk, 4 to 5 years old	22	60	35	4.45	0.04	0.31	3.9–5.1
5	Cows in milk, 6 years and over	17	97	30	4.11	0.07	0.40	3.2–5.0
6	Cows giving 10 to 24 pounds milk	20	71	18	4.40	0.06	0.42	3.7–5.3
7	Cows giving 25 to 40 pounds milk	30	54	31	4.37	0.04	0.36	3.7–5.1
8	Cows giving over 40 pounds milk	14	66	54	4.21	0.07	0.39	3.2–4.8
9	All milking cows of this herd	64	62	32	4.33	0.04	0.49	3.2–5.3
	Herds 1 and 2 combined							
10	Calves 1 to 7 months old	26	4.2	..	6.42	0.06	0.53	5.4–7.4
11	Calves 8 to 13 months old	16	9.8	..	6.01	0.10	0.58	5.2–7.1
12	Calves 14 to 19 months old	20	16.1	..	5.36	0.08	0.52	4.1–6.0
13	Calves 20 to 24 months old	16	22.1	..	4.94	0.05	0.31	4.4–5.6
14	Calves 25 to 30 months old	23	27.0	..	4.70	0.06	0.42	4.0–5.6
15	All milking cows	90	60	33	4.28	0.03	0.42	3.2–5.3
16	All dry cows	17	77	..	4.35	0.06	0.36	3.6–4.9
17	All cows	111	63	..	4.29	0.03	0.42	3.2–5.3

('33); and other workers (Van Landingham, Henderson and Bowling, '35; Eckles, Gullickson and Palmer, '32; Groenewald, '35) conclude that lactation lowers the level of inorganic phosphorus in the blood.

As a normal value for inorganic phosphorus in the blood of milking cows the average for such cows in herd 2 is considered reliable. This value, 4.33 mg. $\pm$ 0.04 mg., with a standard deviation of 0.59 mg. per 100 ml., represents sixty-four animals, among which were representatives of all the common dairy breeds. These cows were giving from 10 to 72 pounds of milk daily. The value for dry cows is similar.

TABLE 2

The significance of the differences in the inorganic phosphorus contents of the whole blood of groups of dairy cattle[1]

GROUP	DESCRIPTION	MEAN DIFFERENCE	P.E. OF DIFFERENCE	ODDS OF SIGNIFICANCE
		Milligrams per 100 ml.		
2 vs. 1	Dry cows vs. milking cows, herd 1	0.279	0.094	22:1
9 vs. 1	Milking cows, herd 2 vs. herd 1	0.227	0.075	23:1
4 vs. 5	Middle aged vs. old cows, herd 2	0.339	0.079	260:1
10 vs. 11	1- to 7- vs. 8- to 13-month old calves	0.402	0.121	39:1
11 vs. 12	8- to 13- vs. 14- to 19-month-old calves	0.653	0.125	2300:1
12 vs. 13	14- to 19- vs. 20- to 24-month-old calves	0.416	0.093	395:1
13 vs. 14	20- to 24- vs. 25- to 30-month-old calves	0.248	0.078	28:1

[1] The first of the two groups in each comparison has the higher value.

The values for the age groups of calves (mostly heifers), as given in table 1, represent 101 animals and should be fairly reliable for immature cattle under winter conditions.

SUMMARY

Inorganic phosphorus was determined in the whole blood of over 200 cattle—mostly cows and heifers—in normal states of health and nutrition.

The inorganic phosphorus of the blood of immature cattle diminished with increase in age in an approximately regular manner.

As to the effect of age on inorganic blood phosphorus values of milking cows, no such effect was observed as between cows 2 to 3 years old compared with cows 4 to 5 years

old, but aged cows had significantly less inorganic phosphorus in their blood than did middle aged cows.

The effects of lactation on inorganic blood phosphorus were questionable.

No influences of the breed of cow or of the kind of roughage consumed on inorganic blood phosphorus values, were observed.

The average inorganic phosphorus content of the blood of sixty-four milking cows was 4.33 mg. $\pm$ 0.04 mg., with a standard deviation of 0.59 mg. per 100 ml.

The corresponding value for dry cows was approximately the same.

ACKNOWLEDGMENT

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THE FACTOR I (VITAMIN B_6) REQUIREMENT OF THE RAT

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THREE FIGURES

(Received for publication September 19, 1938)

The vitamin B complex has now been separated into five distinct components: thiamin, riboflavin, nicotinic acid amide, factor I (vitamin B_6), and factor II (filtrate factor). Of these, thiamin (Williams and Cline, '36), riboflavin (Euler, et al., '35), and nicotinic acid amide (Elvehjem, et al., '37) have been available in crystalline form for some time. With the availability of factor I in crystalline form (Lepkovsky, '38), it became possible for the first time to determine the rat's requirement for this vitamin. Accordingly five groups of rats were fed various levels of crystalline factor I. The levels chosen were 50, 20, 10, 3 and 1 micrograms daily, and each group included a minimum of five animals.

EXPERIMENTAL

Twenty-one-day-old female rats of the Long-Evans strain were placed in cages with raised screened bottoms to minimize the effect of coprophagy. The basal diet consisted of sucrose 59, extracted casein [1] 27, fat (primex) 8, cod liver oil 2, and salt mixture no. 185 (McCollum and Simmonds, '18) 4. Water bottles attached to the cages provided fresh water at all times. All rats were observed daily and weighed at weekly intervals.

[1] The casein was extracted with 20% iso-propanol. Repeated extractions were made until the washings from the casein were entirely free of color. This required ten individual extractions. After the completion of the iso-propanol extractions, the casein is given two washings with acetone; and then dried.

For the first 2 weeks immediately after weaning, the rats were fed the basal diet but no supplements. At the end of the period, the rats were transferred to individual wire-screen floored cages, and the basal diet was supplemented with 25 micrograms each of thiamin and riboflavin daily. These supplements were measured out into Syracuse watch glasses and placed in the cage. The rats became rapidly conditioned to this feeding procedure and avidly consumed the dose as soon as it was placed in the cage. The above dietary regime was maintained for 2 weeks, at the end of which period

Fig. 1 Crystalline factor I. × 290.

factor II (daily dose 0.5 cc.) was supplied as a beef liver extract prepared according to the method described by Lepkovsky, Jukes and Krause ('36). When growth ceased and dermatitis appeared, crystalline factor I was fed. Crystalline factor I was prepared from rice bran extract by the method of Lepkovsky ('38).[2]

The factor I crystallized as needles, largely in the form of rosettes. They formed on the stirring rod, and on the sides and bottom of the beaker. A small amount of gummy material had settled on the bottom of the beaker. The crystals were loosened, and by careful stirring were suspended in the

[2] We wish to thank Doctor Lepkovsky for making available to us the manuscript describing the method for preparing factor I while it was still in press.

alcohol-acetone mixture and poured out quickly to remove a minimum of the gummy material. The dry crystals so obtained had a slight yellowish tinge. The gummy layer in the bottom of the beaker and the crystals remaining behind were dissolved in a minimum of water and again precipitated with alcohol and acetone, and another smaller crop of crystals was obtained. About 140 mg. of crystalline factor I were thus obtained from about 1 liter of the crude eluate. Figure 1 shows the form of the crystals.

Adequacy of factor II was checked on chickens and on rats, the method of Jukes ('37) being used for the former and the procedure of Lepkovsky, Jukes and Krause ('36) being followed for the latter. That the liver extract was free of factor I is evidenced by the cessation of growth and the appearance of dermatitis in rats fed the basal diet supplemented with all the known water soluble vitamins exclusive of factor I.

Results obtained with different levels of factor I

Figure 2 shows the growth secured with different levels of factor I. At 50, 20 and 10 microgram levels there was an immediate resumption of growth and a marked improvement in the dermatitis. Even in cases of the most florid type of dermatitis, involving the ears, paws and face, complete healing occurred within 10 days so that the rats were indistinguishable from normal animals.

At the 1 microgram level, very little growth was made and healing of the dermatitis was very slow. On the 3 microgram level, the healing was much more marked and in some cases almost complete. While 1 and 3 micrograms of factor I were insufficient to bring about a complete cure, some growth took place, and the life of the rats was greatly prolonged.

The survival period of rats deprived of factor I rarely extends beyond 50 to 60 days.

The effect of inanition in factor I deficiency

Since the water-soluble vitamins tend to affect appetite, it seemed of interest to compare the growth responses of animals fed the factor I-free diet and allowed all the food desired, with the growth responses of rats fed the basal diet including factor I but kept on a restricted food intake.

Forty rats consisting of twenty pairs of litter mates were divided into two groups at the time of weaning. One group

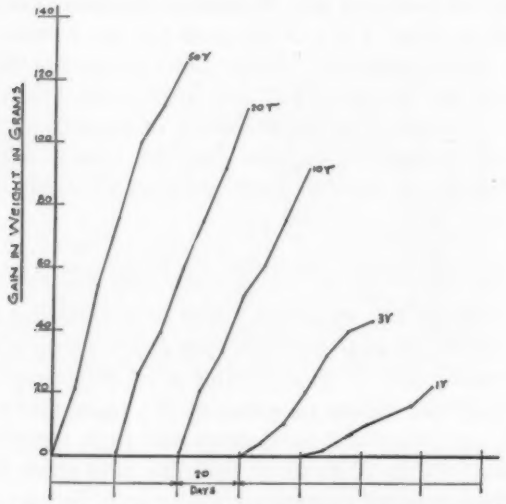

Figure 2 shows the gain in weight in grams of groups of rats fed various levels of crystalline factor I. The levels are 50, 20, 10, 3 and 1 micrograms. Each group includes a minimum of five rats.

was placed on the factor I-deficient regime. The food for each animal of this group was weighed separately each day. Each animal of the second group was fed the average daily ad libitum food intake of the first group plus 20 micrograms of factor I. A third group of rats receiving 20 micrograms of factor I and food ad libitum was included as a positive control group. Figure 3 shows the average growth of these three groups of animals.

The animals on the restricted food intake and receiving twenty micrograms of factor I showed a voracious appetite, finishing the allotted ration within a few minutes after it was placed in the cage.

The average amount of food consumed daily by the groups of deficient animals varied from 4.5 to 4.9 gm. The severity of the dermatitis seemed to have no correlation with the food intake. With the same average food intake in the two groups, 20 micrograms daily of factor I resulted in a heavier body

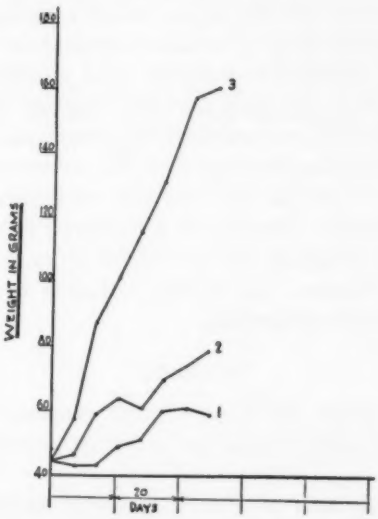

Figure 3 shows the results obtained with three groups of rats as follows: factor I-deficient group, a limited food control group, and a normal control group. Curve 1 shows typical depletion curve for factor I. These rats received the basic diet unsupplemented for 2 weeks; 25 micrograms of thiamin and 25 micrograms of riboflavin were then added, and at the end of an additional 2 weeks 0.5 cc. liver filtrate was added. Curve 2 shows the growth obtained with the same supplements as shown in curve 1 plus 20 micrograms of factor I except with the food limited to the average amount consumed by the factor I-deficient rats. Curve 3 shows the growth obtained when all the vitamin B complex factors are supplied as follows: 25 micrograms of thiamin, 25 micrograms of riboflavin, 20 micrograms crystalline factor I, and 0.5 cc. liver filtrate. These rats received an unlimited food supply.

weight in the limited group. In several cases individual factor I-depleted rats had a larger food intake than some of the animals maintained on the restricted food intake plus factor I,[3] but in spite of this difference weighed less than the latter. Aside from stunting, outwardly the rats on the limited food intake were indistinguishable from normal animals.

The three groups of rats were sacrificed on the seventieth day of life or 49 days after the initiation of the experiment. One outstanding finding was the complete atrophy of the thymus in the factor I deficient group. In the restricted food intake group these glands were present though reduced in size. The ovaries showed no development in either group. The lack of ovarian development was consistent with the observation that in no case, was the vaginal introitus established at the time the experiment was terminated.

Another interesting finding was the presence of considerable amounts of fat in the animals maintained on the restricted food intake. The factor I-deprived rats, on the other hand, showed a complete absence of fat storage. This finding is additional evidence that factor I makes for a greater or more efficient food utilization.

SUMMARY

1. There is slight but recognizable response of rats on a factor I-deficient diet to as little as 1 microgram of crystalline factor I.

2. Ten micrograms of factor I nearly satisfied the rat's requirements for this vitamin.

3. Rats receiving factor I but limited in their food intake to that of rats on the factor I-deficient diet appeared to utilize food more efficiently. This was evidenced by a greater body weight and the presence of fat in their tissues.

4. In the rats on the factor-I deficient diet, the thymus atrophies more quickly than in those receiving the same amount of food with factor I.

[3] It should be recalled that rats receiving factor I were fed the average daily food intake of the entire group of deficient animals.

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THE EFFECT OF YEAST AND OF THIAMIN ON THE PRODUCTION OF LOW IODINE GOITER [1]

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(Received for publication September 15, 1938)

Carpenter and Sharpless ('37) have recently reported rather startling findings concerning a heat labile factor, not vitamin B_1, in dried brewer's yeast capable of decreasing the size and increasing the iodine concentration of the thyroid glands of rats on a low iodine, goiter producing diet. In three groups of rats all receiving diets of equal iodine content (0.0038%) one group was fed 5% dried brewer's yeast, the second group 5% autoclaved yeast and the third 5% autoclaved yeast plus vitamin B_1 as a wheat germ concentrate. The results showed that the first group, which had received the untreated yeast, had smaller thyroids and considerably more thyroid iodine than did the animals in the other two groups.

These same workers also concluded that the goiter observed in animals on a low iodine, vitamin B_1-deficient diet can be prevented either by iodine or by vitamin B_1. The vitamin B_1-free diet containing 0.0038% iodine which they used, produced definite thyroid enlargement and a histological picture of goiter whereas if this diet were supplemented either with iodine or with a vitamin B_1 concentrate, the thyroids were smaller in size and histologically normal.

The experiment reported here was set up to test the conclusions of Carpenter and Sharpless by a somewhat different mode of attack. Young rats, averaging 75 gm. in weight, were

[1] Aided by a grant from the Committee on Scientific Research of the American Medical Association.

placed on a low iodine, vitamin B_1-deficient diet for a preliminary period of 7 days. At this time all of the animals had ceased to grow and began to lose weight. They were divided into groups of ten, equally distributed as to sex, placed on the diets shown in table 1, and continued on experiment for 6 weeks, a period similar to that used by Carpenter and

TABLE 1

	GROUP			
	I Low-iodine adequate B_1 (yeast)	II Low-iodine vitamin B_1-free	III Low-iodine adequate B_1 (thiamin)	IV Adequate iodine vitamin B_1-free
Diet				
Sucrose (%)	68	68	68	68
Wheat gluten (%)	18	18	18	18
Dried yeast (%)	5	..	..	..
Autoclaved yeast (%)	..	5	5	5
Pig liver (%)	2	2	2	2
Salt mixture no. 351 (%)	2	2	2	2
Cottonseed oil (%)	5	5	5	5
Iodine (γ %)	..	..	..	50
Vitamin supplements (daily)				
Carotene (IU)	4	4	4	4
Viosterol (IU)	4	4	4	4
Thiamin (γ)	..	..	15	..
Results				
Weight gain (gm.)	79.9	—18.1	72.3	—8.2
Thyroid weight (mg.)	20.5	8.2	17.2	7.8
Thyroid weight per 100 gm. body weight (mg.)	13.0 ± 0.52	12.8 ± 0.41	11.4 ± 0.23	11.0 ± 0.39
Dry matter (%)	23.6	23.3	22.2	35.2
Iodine, dry basis (%)	0.049	0.088	0.072	0.283

Sharpless. The technic devised by Remington ('37) was then used, wherein the status of the thyroid gland is quantitatively measured by the determination of three criteria: weight of gland in relation to body weight, dry matter and iodine content of the gland. The results are shown in table 1.

The animals of groups II and IV all showed polyneuritic symptoms typical of vitamin B_1 deficiency at least once during

the experimental period. Each time these symptoms occurred the animal was given sufficient thiamin (6 γ) orally to relieve the paralysis and allow a slight gain in weight for approximately 5 days. In this manner the animals were all maintained on their respective diets the same length of time.

It might be well at this point to note that although the basal diet used by Carpenter and Sharpless reputedly contained less than 20 γ of iodine per kilogram yet their values for size, dry matter and iodine content of glands of rats maintained on this diet were within the normal range as determined by Remington, Remington and Welch ('37). The inference must be drawn that these animals had received prophylactic amounts of iodine from some source. Furthermore, the diets to which iodine was added furnished prodigious quantities of this element in relation to the minimal protective dose of 1 γ to 2γ per day as established by Levine, Remington and von Kolnitz ('33). Assuming an average daily food consumption of 10 gm. the diets containing 0.0038% and 0.019% iodine would supply 380 γ and 1900 γ per rat per day or from 200 to 1000 times the amount necessary for normal thyroid development. Apparently these investigators were in no case dealing with low iodine goiter. Their histological sections tend to confirm this opinion.

In the present experiment at autopsy the thyroid glands of all group IV animals (those receiving the adequate iodine, vitamin B_1-free diet) were obviously normal on gross inspection, viz., very pale in color and firm in texture. In contrast, the glands from all the animals in the other groups were definitely goitrous in that they were apparently enlarged and were dark red in color with considerable engorgement and distension of the blood vessels leading to the gland. The iodine deficiency in the diets employed, while producing definite changes in the dry matter and iodine content of the glands, was not sufficiently drastic to cause significant increases in thyroid weights (Remington and Levine, '36).

It is apparent, therefore, under the conditions of the present experiment, that dried brewer's yeast is ineffective

in influencing the size or iodine content of thyroid glands of rats fed a low iodine, goiter producing diet. The yeast fed rats possessed thyroids equal in size, dry matter and iodine content to those of the animals receiving autoclaved yeast or autoclaved yeast plus thiamin.

Furthermore, the data show clearly that vitamin B_1 cannot substitute for iodine in preventing low iodine goiter. Thyroids of rats on a low iodine, vitamin B_1-free diet supplemented by minimal amounts of iodine (approximately 2 to 3 γ per day) proved to be normal in every respect while the glands of rats on the same diet supplemented by vitamin B_1 (15 γ thiamin daily) presented a picture of true goiter. The glands differed in no way from those of animals given the same basal diet with no vitamin B_1 supplement. The percentage of dry matter was low, the iodine content was low and the macroscopic appearance was typical of hyperplasia.

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THE ACID SOLUBLE PHOSPHORUS CONTENT OF MUSCLE OF RATS UNDER VARIOUS DIET MODIFICATIONS [1]

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ONE FIGURE

(Received for publication September 12, 1938)

Previous reports from this laboratory have indicated that the content of acid soluble phosphorus in muscular tissue of the rat is not constant over the life of the animal (Struck and Visscher, '35; Bartoli, Cohen and Struck, '37). Numerous other chemical changes relating to growth and age have appeared in the literature reviewed by Pincussen, Reed and Visscher ('38) in a report on the content of acid soluble phosphorus in human tissues at various ages.

The report by Struck and Visscher of a decrease in the content of acid soluble phosphorus in old animals was partially confirmed in another colony that was maintained for 11 months and sampled at regular intervals. However, since certain confusing factors cast some doubt on the conclusions, a third large colony was maintained for 65 weeks.

Since there is no clear means of differentiating between the chemical changes incident to growth and those due to aging, an attempt was made to determine whether factors that might influence growth in either direction would have any influence on the concentration of acid soluble phosphorus. The animals were Wisconsin strain white rats, 3 weeks old when placed on the stock laboratory diet.

[1] Part of the expenses of this investigation was borne by grants from the Wander Company, from the Nutrition Research Laboratories and from the Graduate School Research Fund of the University of Illinois.

At the age of 13 weeks the animals were placed on the various special diets noted below. A group of ten animals, selected at random from the colony, was killed at this time and analyses for total acid soluble phosphorus made on gastrocnemius muscle and liver. Seven groups were disposed as follows:

Group 1. Controls: stock diet only. Ca and P content 0.5 to 0.6% with Ca: P ratio approximately 1.

Group 2. High phosphorus diet. Sufficient disodium phosphate was added to the stock diet to increase the phosphorus content to 1.25 to 1.50%. The Ca: P ratio varied from 0.33 to 0.41.

Group 3. Stock diet fortified with vitamin B_1 [2] in the form of Tiki-tiki extract.

Group 4. Diet 2 with the same vitamin B_1 supplement as for group 3.

Group 5. Stock diet; growth hormone [2] administered intraperitoneally.

Group 6. Diet 2, with the same dosage of growth hormone as for group 5.

Group 7. Diet 4, with the addition of vitamin D of such concentration that the daily dose, given by stomach tube, was 2½ units per rat.

The rats were kept on the various diets from the age of 13 weeks to the age of 65 weeks, when the experiment was terminated. Analyses for total acid soluble phosphorus of muscle and liver were made on all animals. The results were shown in table 1 and figure 1. At 43 weeks of age ten rats from each group were killed and analyzed.

The technic for analyses was: The rats were anaesthetized with ether, the gastrocnemii and liver were quickly excised and immediately dropped into liquid air. Weighed samples of muscles and liver were ground to powder with mortar and pestle cooled with liquid air, and the powder then extracted with a measured quantity of 0.25 N trichloracetic acid.

[2] Antuitrin Growth was generously supplied by Parke, Davis and Company; vitamin B_1 in the form of Tiki-tiki extract, by the Harris Laboratories.

Aliquot parts of the filtered extract were ashed in micro-Kjeldahl flasks with concentrated H_2SO_4 and 30% H_2O_2. After boiling with water to decompose excess H_2O_2, phosphorus was determined by a modified Bell-Doisy method.

The growth curves showing the influence of the dietary modifications were omitted in order to conserve space, but

TABLE 1

Total acid soluble phosphorus muscle (moist tissue), third colony

AGE	MEAN P CONTENT	STANDARD DEVIATION	STANDARD ERROR
	mg.%		
Group 1			
3 months	125	9.92	3.14
10 months	155	3.95	1.49
16 months	113	14.60	4.40
Group 2			
3 months	125	9.92	3.14
10 months	159	6.91	2.44
16 months	129	10.07	3.36
Group 3			
3 months	125	9.92	3.14
10 months	156	8.04	2.84
16 months	137	13.55	3.29
Group 4			
3 months	125	9.92	3.14
10 months	146	12.10	4.03
16 months	132	12.87	4.86
Group 5			
3 months	125	9.92	3.14
10 months	144	6.83	2.41
16 months	143	13.90	4.64
Group 6			
3 months	125	9.92	3.14
10 months	148	10.23	3.41
16 months	132	15.39	5.13
Group 7			
3 months	125	9.92	3.14
10 months	143	13.76	4.59
16 months	126	19.76	6.25

will be made available to anyone interested. In all groups the growth rate was inhibited compared with the controls, except in those receiving growth hormone.

The values obtained for the phosphorus content of the muscles of rats of the third colony are shown in table 1. Probability factors were calculated according to the method given by Fisher, but are not included for lack of space.

Data were also obtained for total acid soluble phosphorus content of liver at the same intervals, but are not included since they did not show consistent changes. In general, the

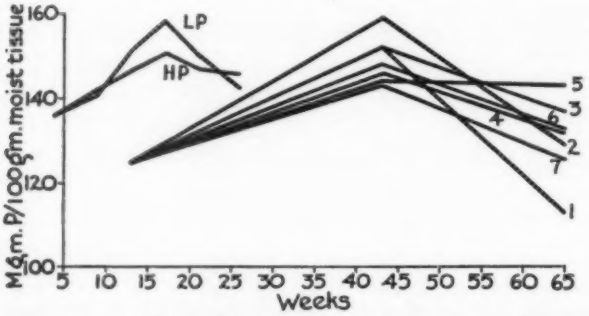

Fig. 1 L.P. (low P diet) and H.P. (high P diet) represent changes in acid-soluble P in muscle of rats in colony IV. Graphs 1 and 7 inclusive represent changes in acid soluble P in muscles of rats in colony III. 1, normal controls; low P diet. 2, high P diet. 3, no. 1 diet + vitamin B_1. 4, no. 2 diet + vitamin B_1. 5, no. 1 diet + growth hormone. 6, no. 2 diet + growth hormone. 7, no. 4 diet + vitamin D.

results for the livers followed the same trend as the muscles, but gave changes which were significant with considerably less regularity than the muscles.

The analytical data were then studied in an effort to correlate the changes in phosphorus content with the various diets. The results of this study are not included here, but may be summarized briefly.

In all groups the acid soluble phosphorus content of muscle increased up to 42 weeks and decreased thereafter except in group 5 (low P diet + growth hormone). The differences at 43 weeks were not statistically significant. At 65 weeks, the

differences were significant between groups 1 and 5. Apparently the growth hormone retards the decrease in acid soluble phosphorus on a low P diet.

Cole and Koch ('31) have studied the phosphorus fractions of striated muscles of rats and have found that there is a rapid increase in creatine phosphate content from birth to 2 or 3 weeks after weaning, a change which may be due to the relatively great increase in exercise during this period. They did not find any significant change from the maximum during a total period of observation up to the 168 days of age.

It seemed possible that this early increase had been missed in our series. In an effort to check this point another series of experiments was carried out on a fourth colony. The rats were obtained from the same source as those in the other experiments. At weaning they were placed on the stock diet as above for 1 week, after which ten of each sex were selected at random and basic control analyses were made. Half of those of each sex were then placed on a diet prepared by adding disodium phosphate to the stock diet in quantity sufficient to bring the phosphorus content to 1.25 to 1.5%.

As already described, analyses of the muscles of the rats were made on ten males and ten females of each group at the ages of 28, 60, 90, 120, 150 and 180 days.

The growth curves of the animals of the fourth colony showed that the high phosphorus diet caused a definite retardation of growth in males, although not in the females. The results of the analyses are summarized in table 1 (fourth colony) and figure 1. The acid soluble phosphorus contents of the muscles of rats on both diets, when calculated as per cent of moist tissue in both groups increased significanty to a maximum at 120 days of age then slowly decreased, the final value at 180 days being higher than the control at 28 days.

Tables showing comparison of water, acid-soluble phosphorus and nitrogen content of the muscles of the two groups at monthly intervals were prepared, but are omitted for lack of space.[3] It was observed that there are significant differ-

[3] Complete data are available for those interested in details.

ences between the two groups only in random instances, leading to the provisional hypothesis that during the first 6 months of life the addition of sodium phosphate to the diet in the amounts used here has no effect on these constituents.

In figure 1 the two small curves labeled L.P. and H.P. confirm Cole and Koch in their report of an early increase in the muscle content of acid soluble phosphorus. However,

TABLE 2

Total acid-soluble phosphorus content of muscles of rats (moist tissue), fourth colony

AGE	DIET	MEAN PHOS.	NUMBER OF ANALYSES	STANDARD DEVIATION	DIFFERENCE [2]
days		*mg.%* [1]			
28	SD	136	17	9.6	..
60	SD	141	15	9.9	5
	HP	142	16	11.5	6
90	SD	151	14	9.4	15
	HP	147	13	9.2	11
120	SD	158	15	6.6	22
	HP	151	14	7.7	15
150	SD	150	15	9.1	14
	HP	147	17	5.7	11
180	SD	143	17	5.8	7
	HP	146	18	7.6	10

SD = stock diet, Ca: P ratio 1.
HP = high phosphorus diet.

[1] Includes values for right and left muscles and for males and females.
[2] Differences from value at 28 days of age.

there then occurred a decrease which placed the values at the end of 25 weeks somewhere near those for groups 1 and 2 and in the third colony, as shown in the same figure. However, the values in these earlier observations were unquestionably increasing. Furthermore, the initial determinations made at 13 weeks of age were very much lower than occurred at any time in the fourth colony. Both lots were treated similarly and were of the same strain. The only explanation

that can be offered at present is that there is a seasonal influence. All rats in the third colony were born August 18, 1935. Those in the fourth colony were born March 18, 1937. It will be necessary to investigate further the possible seasonal influence.

SUMMARY

1. The acid soluble phosphorus content of skeletal muscle of white rats in the third colony on all dietary modifications used showed a marked increase from 13 weeks to 43 weeks.

2. The high phosphorus diet produced the greatest increase. The other diets retarded the increase when compared to the normal diet, but the differences were not statistically significant.

3. From 43 to 65 weeks there was a definite decrease in acid-soluble phosphorus content which was most pronounced on the normal diet and definitely retarded in all other groups. Normal diet plus growth hormone prevented the decline practically completely. Again differences were not statistically significant except in this one instance.

4. In the fourth colony there was found an increase in the acid soluble phosphorus content of muscle up to 17 weeks, which was retarded in this case by the high phosphorus diet. From 17 to 25 weeks the decrease found on the normal diet was retarded by high phosphorus diet.

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THE EFFECTS OF HIGH HUMIDITY ON SKIN TEMPERATURE AT COOL AND WARM CONDITIONS [1]

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The importance of humidity in thermal reactions of the body has never been satisfactorily evaluated. Yaglou ('37) states that "under ordinary indoor conditions during the heating season, variations in humidity are relatively unimportant" and that "practically all physical, physiologic and psychologic tests have proved to be negative or inconclusive." Wiley and Newburgh ('31) found no change in skin temperature when subjects were exposed to a relative humidity varying from 20 to 80% at a room temperature of 28°C. In ten subjects exposed to ordinary room temperatures (20 to 22.5°C.) Talbot ('31) observed no consistent changes in skin temperature when the relative humidity varied from 31 to 90%. Winslow, Herrington and Gagge ('37) noted that skin temperatures were in general the same for both low and high humidities. On the other hand, Muira ('31) observed that the skin temperature of the forehead and over the carotid artery tended to vary directly with the relative humidity of the air at temperatures between 60° and 80°F. although the variation was small. In addition, Phelps and Vold ('34) noted a slight positive correlation between the temperature of the forehead and cheeks and the relative humidity during the closed window season.

In view of the inconsistency in results of previous researches the subject was investigated in normal individuals as

[1] This investigation was aided by a grant from the Rockefeller Foundation.

a part of a physiologic study of thermal reactions from the standpoint of their autonomic reactivity.

The study was carried out on ten normal male subjects who lived at the institution throughout the duration of the tests. On each experimental day each participant came from his bed under fasting conditions to the temperature laboratory where he disrobed and lay down upon a cane-woven bed without any coverings. Immediately after reclining and every 30 minutes thereafter over a period of 3 hours, measurements were taken of the skin temperatures thus giving a total of seven series of readings. Skin temperatures were determined at ten representative areas on the right anterior surface of the body, one side being considered sufficient because of the high degree of bilateral symmetry of skin temperatures (Freeman, Linder and Nickerson, '37). All readings were taken by means of copper-constantin thermocouples.

The effects of humidity were studied by a comparison of the levels of skin temperatures observed on 2 days at the same environmental temperature, the first day having a constant low humidity of 20% and the second day a relative humidity which increased from 20 to 90%. The procedure was carried out at two environmental temperatures, 24°C. and 32°C. conditions which are ordinarily characterized by a cooling of the skin and a warming, respectively. For the purposes of this experiment, therefore, 4 days were utilized. The actual environmental conditions for the days with changing humidity are shown in table 1. On each humid day the first two series of measurements were made under the same conditions as on the previous dry day in order to be sure that the subjects started at approximately the same level. The rate of increase in relative humidity on the 2 humid days is quite similar so that a comparison of the results obtained at the different temperatures is possible. The air velocity is essentially the same for the 4 days, the average level being 41 feet per minute.

The behavior of the skin temperatures at 24°C. and 20% R.H. are shown in table 2 in the columns headed 24°, 'Dry.' Here are tabulated the means of the ten subjects for the seven series of measurements at the various skin points. Since they serve as a basis for a comparison of the data obtained on the humid days, they are presented in full though in themselves they show little that is new. In summary, it may be said that there is a downward trend in all the skin temperatures, the fall being more marked and prolonged on the extremities. A stable level is reached on the trunk after 1 hour of exposure and on the extremities 1½ hours later.

TABLE 1

Means of levels of relative humidity attained at 30-minute intervals at environmental temperatures of 24°C. and 32°C.

Time of exposure Minutes	*Relative humidity (D.B. 24°C.)* %	*Relative humidity (D.B. 32°C.)* %
0	22.8	19.1
30	22.4	21.3
60	49.6	47.9
90	77.6	71.8
120	87.7	86.9
150	90.0	90.4
180	90.5	90.2

As the relative humidity is increased from 20%, after the first 30 minutes of exposure there is observed a divergence of trends from those seen under conditions of constant low humidity so that the fall in skin temperature is markedly lessened. This results in the values for skin temperature being higher than those obtained at the corresponding time at 20% R.H. The difference in effects is shown in table 2 in the columns marked 24° 'Humid.' The first two readings on the 'dry' and 'humid' days are obtained under the same environmental conditions. The slight deviations present are probably due almost entirely to the innate variability of the organism. The third reading on the 'humid' day at which the relative humidity has been increased to 50% begins to show a difference in effect from that of the corresponding value at

TABLE 2

Mean skin temperatures (0°C.) of ten normal subjects at environmental temperature of 24°C. with high and low humidity and 32°C. with high and low humidity

			TIME OF EXPOSURE IN MINUTES							AVERAGE DIFFERENCE BETWEEN TERMINAL READINGS
			0	30	60	90	120	150	180	
Forehead	24°	Dry	31.9	32.4	32.4	32.2	31.9	31.8	31.9	0.5
		Humid	31.9	32.7	32.7	32.8	32.6	32.6	32.4	
	32°	Dry	33.5	34.6	34.6	34.8	34.9	35.0	34.9	0.2[1]
		Humid	33.7	34.8	35.2	35.4	35.3	35.3	35.1	
Chest	24°	Dry	31.9	31.5	31.3	31.1	31.2	31.2	31.3	0.4
		Humid	32.0	31.7	31.7	31.9	31.7	31.7	31.7	
	32°	Dry	33.7	34.2	34.3	34.6	34.6	34.8	34.8	0.1[1]
		Humid	33.7	34.5	34.9	35.1	35.1	35.0	34.9	
Abdomen	24°	Dry	32.6	32.0	31.8	31.5	31.5	31.5	31.4	0.3
		Humid	32.8	32.2	32.0	32.0	32.0	31.9	31.7	
	32°	Dry	34.1	34.3	34.3	34.5	34.7	34.7	34.7	0.2[1]
		Humid	34.2	34.5	34.8	35.1	35.0	34.9	34.9	
Upper arm	24°	Dry	32.4	32.0	31.6	31.4	31.2	31.1	31.0	0.5
		Humid	32.5	32.1	31.9	31.9	31.8	31.7	31.5	
	32°	Dry	34.0	34.3	34.3	34.6	34.7	34.8	34.7	0.2[1]
		Humid	34.0	34.5	35.0	35.2	35.1	35.1	34.9	
Lower arm	24°	Dry	32.2	31.6	31.2	30.5	30.2	29.9	29.8	0.9
		Humid	32.3	31.9	31.5	31.2	31.0	30.9	30.7	
	32°	Dry	33.9	34.1	34.3	34.5	34.6	34.6	34.7	0.4
		Humid	33.7	34.3	34.9	35.2	35.3	35.2	35.1	
Thumb	24°	Dry	28.5	27.7	27.3	26.5	25.9	25.6	25.5	2.2
		Humid	29.4	28.4	28.0	27.9	28.0	27.8	27.7	
	32°	Dry	31.7	33.3	33.7	33.4	33.6	33.6	33.5	1.8
		Humid	31.4	33.9	34.8	35.3	35.4	35.3	35.3	
Finger	24°	Dry	23.9	23.9	24.4	23.4	23.1	23.0	22.9	3.3
		Humid	25.6	25.2	24.9	25.8	26.4	26.4	26.2	
	32°	Dry	29.0	32.9	33.4	32.9	33.0	32.8	32.9	2.6
		Humid	29.6	33.5	34.9	35.4	35.5	35.6	35.5	
Thigh	24°	Dry	31.3	30.8	30.5	30.3	30.1	29.9	29.9	0.6
		Humid	31.6	31.0	30.9	30.9	30.8	30.7	30.5	
	32°	Dry	33.0	33.7	33.9	34.0	34.1	34.2	34.2	0.5
		Humid	33.3	34.0	34.5	34.8	34.8	34.8	34.7	
Lower leg	24°	Dry	31.3	30.5	30.0	29.6	29.4	29.2	29.2	0.9
		Humid	31.5	30.9	30.6	30.6	30.4	30.2	30.1	
	32°	Dry	33.1	33.6	33.6	33.8	34.0	34.0	34.0	0.5
		Humid	33.4	33.9	34.2	34.6	34.6	34.5	34.5	
Foot	24°	Dry	28.7	27.4	26.8	26.2	25.7	25.5	25.2	2.0
		Humid	29.5	28.3	27.9	27.8	27.7	27.5	27.2	
	32°	Dry	31.1	31.1	31.3	31.3	31.4	31.2	31.2	3.4
		Humid	30.7	31.2	32.3	33.6	34.3	34.6	34.6	

[1] Denotes non-significance.

20%, a variation which becomes more marked as the humidity increases. The maximum deviation from the corresponding value at 20% is obtained on the trunk when the relative humidity has reached 78% (fourth reading) after which there is a slight decline in the difference. On the hand, the effect of the increased humidity is much more marked than on the trunk and reaches a maximum difference at the sixth reading where the relative humidity is 90% after which the deviation from the corresponding value at 20% relative humidity is maintained.

It is evident, then, that the effect of increasing the humidity is to offset to a varying degree the cooling ordinarily observed at the same environmental temperature with a low humidity. This diminution of heat loss is noticeable when the realtive humidity has been increased to only 50% and reaches a maximum at higher levels of humidity, 78% on the trunk and 90% over the rest of the body. Following this increase in skin temperature, the thermodynamic situation is changed, and as a result of further loss through the channels of radiation and convection, the surface of the trunk and upper extremities tends to cool despite the maintenance or even increase of the high level of humidity. The difference in trends between the dry and humid days at 24° have been analyzed by the method of the analysis of variance and have been found to be statistically significant at all points but the abdomen.

A quantitative estimation of the effects of humidity on skin temperature is shown in table 2 in the column containing the differences between the terminal readings. For this analysis the averages of the last series of measurements on the dry and humid days have been taken in order to afford a comparison of the skin and body temperatures at 20% and 90% relative humidity after a sufficient period of exposure to allow thermal equilibrium to take place. It is evident in all instances that the skin temperatures are higher at 90% relative humidity than at 20%. These differences are all statistically significant. The average elevation of skin temperature

varies topographically on the body surface, the extremities showing a much greater effect than the trunk. It should be noted that these differences should not be taken at their exact numerical value in view of the slight variation in the initial readings between the two experimental days and the tendency for the final skin temperatures to decrease slightly on the humid day. Nevertheless the effect of high humidity on skin temperatures is sufficiently marked, particularly on the hands and feet, so that there can be little question as to its physiologic significance.

Turning now to the effect of humidity at the higher temperature, it may be seen that under constant environmental conditions of 32°C. and 20% relative humidity (table 2, 32° 'Dry') the skin temperature rises, slightly on the trunk and markedly on the extremities. The elevation of temperature on the trunk reaches its maximum level in 2 hours while on the extremities, the highest point is attained in 30 minutes. All of the values are, of course, at a much higher level than the corresponding ones at 24°C. and 20% relative humidity.

When the relative humidity is raised, the same phenomenon is observed as at 24°C. The skin temperature again increases all over the body, slightly on the trunk and to a greater extent on the extremities (table 2). This increase is an augmentation of an already existing heating trend rather than lessening of a cooling trend as at 24°C. On the trunk and upper extremities, the effect of humidity reaches its maximum after 90 minutes of exposure at 72% R.H. when as seen at the lower temperature a new equilibrium is established and the magnitude of the increase in skin temperature diminishes. On the hands and feet, however, the effect of humidity reaches its maximum usually when 90% R.H. has been first attained, 2.5 hours after first exposure. The differences in the heating trends between the dry and humid days have been found to be statistically significant by the method of the analysis of variance and in all instances except over the forehead and chest.

The changes in skin temperature at 32°C. as a result of the introduction of high humidity have been computed in the same way as at 24°C. In table 2, it is seen that under the humid conditions the values are again increased with a similar degree of variation over the different parts of the body surface, the extremities again showing the greatest effect. The differences are not significant over the forehead, trunk and upper arms. A comparison of the increases in temperature at 32°C. with those at 24°C. brings forth the fact that the values at the warmer temperature are not higher as Miura ('31) has stated but are even slightly lower. In short, the effects of high humidity on skin temperature are quite similar irrespective of the fact whether the environmental condition is one where there is cooling or warming of the surface of the body. This is true at least within the limits of this study.

A consideration of these data reveals several interesting facts. First, increasing humidity at these air temperatures causes significant elevations in the temperature of the skin. The effect is noted when the humidity is increased from 20 to 50% and becomes more marked as the humidity is increased to 90%. In general, this result may be ascribed to the inhibition of evaporation of perspiration by the increased atmospheric moisture (Wiley and Newburgh, '31) and the negation of the heat loss which ordinarily follows upon this function.

Second, the increase in skin temperature thus observed is much more marked on the extremities than on the trunk. This may be due to two factors. The evaporation per unit surface is ordinarily much greater on the extremities than on the trunk (Kuno, '30). The neutralization of this cooling effect may be expected therefore to produce a greater rise in temperature at the distal portions of the body. A second factor is found in the relationship between the saturation vapor pressures at the temperatures of the skin and of the air.

Third, high humidity at the two environmental temperatures has resulted in increases in temperature which have

been quite similar (or even less at 32°C.) for corresponding points on the skin.

Thermodynamic analysis

Following Winslow, Herrington and Gagge ('37) we analyze the heat exchange of the body into four components. Denoting metabolism by M, the rate of heat loss suffered through radiation and convection by C, the rate of heat loss suffered by the cooling of the tissues by S and the rate of evaporation by E the following equation holds:

$$M + S = E + C. \tag{1}$$

According to the cited authors, metabolism is practically constant. It is reasonable to assume that the data of table 2 were obtained after a state of thermal equilibrium has been reached, so that the term S can be neglected.

While investigating the rate of heat loss through evaporation, we shall consider the skin of the human body as a water surface. However, we have to keep in mind that this is only a rough approximation, which applies only when the humidity is high. The ability of the human body to regulate its heat exchange depends primarily on the fact that it is able to act not as a water surface. When the humidity is low, the evaporation is less than that of a water surface, because only as much sweat is produced as is necessary for the maintenance of a heat equilibrium. This condition is described in the language of Winslow, Herrington and Gagge ('37) by saying that the wettedness of the body is less than 100%. However, if the humidity of the surrounding air is high, the excreted sweat cannot evaporate at once, the body becomes wet and acts like a water surface. Hence the following equation holds:

$$E = k_1 (p_T - p_{T_w}) T, \tag{2}$$

where k_1 is an appropriate constant (depending on the air velocity), T is the absolute temperature of the skin, T_w is the wet bulb temperature and p_T the vapor pressure at absolute

temperature T. It is easy to see without any calculations that if T_w is changed then the percental decrease in E will be much larger when T is near to T_w than otherwise. For small intervals and under 40°C., $p_T - p_{T_w}$ can be assumed to be proportional to $T - T_w$; hence $\frac{\delta E}{\delta T_w}$, the change of evaporation rate for the change of T_w by 1°C. is constant.

If, for example, $T_1 = 33°C.$, $T_2 = 25°C.$ and $T_w = 21°C.$ then the rates of evaporation at the points 1 and 2 compare like 3.9 to 1. The increase of T_w from 21°C. to 22°C. causes a decrease by the same amount in both cases, hence is much more significant for point 2 than for point 1.

As soon as the humidity reaches a high degree, as in the experiment, the evaporation slows down markedly at all points where the temperature is near air temperature. Since heat loss through convection and radiation does not increase unless the temperature of the skin increases, it is to be expected that the points of originally low temperature will warm up most. It happens that these points are on the extremities. The increases in temperature at various points of the body look quite proportional, but this is so because the orginal temperatures on the 2 dry days are similarly distributed. That is, if one point is warmer than the other on the cold dry day, then it is still warmer on the hot dry day. There are, however, exceptions; e.g., the foot point, and in these exceptional cases the proportionality breaks down. However, if we arrange the dry day mean temperatures in the order of magnitude, the correlation between dry day temperature and rise due to humidity is conspicuous.

SUMMARY

The effects of high humidity upon skin temperature were studied in ten normal subjects at environmental temperatures of 24°C. and 32°C. The variation due to humidity was observed by a comparison of the levels of surface temperatures when the subjects were exposed for 3 hours in a nude fasting state to low relative humidities (20%) and to increasing humidity (up to 90%) on 2 days at each environmental

temperature. At 24°C., it was found that the cooling of the skin ordinarily observed at 20% relative humidity was partially inhibited by high humidity so that its temperature rose. At 32°C. the heating of the skin found at 20% relative humidity was increased by high humidity. The rise in temperature over the trunk was slight but on the extremities there was an increase of 2 to 3°C. as the result of increasing the relative humidity to 90%. The increase in skin temperature as the result of high humidity at 32°C. was no greater, in fact slightly less, than at 24°C.

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THE EFFECT OF CHONDROITIN SULFURIC ACID ON GIZZARD EROSION AND GROWTH IN CHICKS

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These investigations were stimulated by the possibility that chondroitin sulfuric acid (hereafter abbreviated as CSA) might prevent gizzard erosions. This theory was based on our observation that CSA exhibited very similar properties to those described for the active portions of lung tissue by Bird, Kline, Elvehjem, Hart and Halpin ('36). Bird and Oleson ('38) later presented a preliminary report on CSA as the anti-gizzard erosion factor. Meanwhile our investigations had indicated that CSA was not effective. The diet used by Bird and Oleson was a modification of that which they had employed in previous studies. Since our results with CSA added to the older gizzard erosion producing diet (454) of the Wisconsin investigators were at variance with their findings when the modified diet was employed, we extended our experiments to include further studies on both the new and old gizzard erosion producing diets of the Wisconsin group, on ordinary commercial chick mash and on the gizzard erosion producing diet of Almquist and Stokstad ('37). In all, about 400 chicks were used in these investigations and the results have been subjected to statistical analysis.

METHOD AND DIETS

Barred Plymouth Rock chicks were used throughout these studies and were placed on the diets when 1 day old. The chicks were wing-banded, kept in temperature controlled brooders in an air-conditioned room, and allowed free access to feed and water. The chicks were individually weighed at 2 and 4 weeks, at which time they were sacrificed for inspection of the gizzards. In some of the first experiments, a group from each lot was sacrificed at earlier intervals. The gizzard erosions were graded by number as follows: 1, none; 2, slight; 3, marked and 4, severe. Statistical analyses were made in each group to determine the significance of weight differences and incidence of erosion. The mean erosion score was determined by adding the gizzard erosion grades for all chicks and dividing by the number; the probable error of the difference between the mean erosion score of the control and supplemented animals was then determined in the usual manner.

The basal diets employed are referred to in this report as Wisconsin diet 454 as given by Bird, Kline, Elvehjem, Hart and Halpin ('36), the new Wisconsin diet used by Bird [1] and Oleson ('38), and that of Almquist and Stokstad ('37) (abbreviated as A & S). When a vitamin K supplement was added to the latter diet, it consisted of a hexane extract of alfalfa meal dried on the basal ration in an amount equivalent to 2% of the original material. The new Wisconsin diet consisted of heated dextrin 64, heated purified casein 18, salts 5, dried brewer's yeast 1, liver extract 2, cod liver oil 1, and alfalfa leaf meal 10.

Both commercial [2] and purified CSA were used. Purification was accomplished by digestion with 2% sodium hydroxide and precipitation with 1 to 1½ volumes of alcohol. The precipitate was then dissolved in water and the CSA reprecipitated with 6 volumes of glacial acetic acid. The commercial product was found to contain about 28% glucuronic

[1] We wish to thank Dr. H. R. Bird for giving us the details of this diet in a personal communication.

[2] Obtained from the Wilson Laboratories, Chicago, Illinois.

acid, the purified material about 33% glucuronic acid. Some protein and salts were present in the commercial product; the purified CSA was a biuret-free powder.

RESULTS

The results of the first experiment in which Wisconsin diet 454 was used and 5% CSA was the supplement, are given in tables 1 and 2.

TABLE 1

Tests 28-W3 and 28-W8. Growth on Wisconsin diet 454 compared with supplement of 5% commercial chondroitin sulfuric acid

AGE OF CHICKS	BASAL DIET		BASAL DIET PLUS 5% CSA		DIFFERENCE OF MEANS	P.E. OF DIFFERENCE
	Number of chicks	Mean weight	Number of chicks	Mean weight		
weeks		*gm.*		*gm.*		
2	38	62.5	35	72.8	10.3	±1.56
3	10	89.9	10	116.3	26.4	±7.4
4	15	125.7	15	138.9	13.2	±6.5
5	14	154.3	10	192.9	38.6	±11.2

TABLE 2

Tests 28-W3 and 28-W8. Incidence and severity of gizzard erosions on Wisconsin diet 454 compared with supplement of 5% commercial chondroitin sulfuric acid

AGE OF CHICKS	BASAL DIET					BASAL DIET PLUS 5% CSA					DIFFERENCE OF MEANS	P.E. OF DIFFERENCE
	Gizzard erosion score [1]				Mean erosion score	Gizzard erosion score				Mean erosion score		
	1	2	3	4		1	2	3	4			
weeks												
2	1	3	5	1	2.6	5	2	1	2	2.0	0.60	±0.31
3	1	4	3	2	2.6	3	5	1	1	2.0	0.60	±0.27
5	3	4	4	3	2.43	2	3	4	1	2.40	0.03	±0.25

[1] Figures show number of chicks receiving each score.

The growth of these chicks (table 1) was significantly improved by the addition of CSA. In each weighing except that carried out on the fourth week, the difference between the mean weights of the chicks on the basal ration and those receiving CSA was statistically significant. It may be noted that growth on the Wisconsin diet 454 was rather poor when compared to that attained on good commercial chick mash.

A deficiency of other factors than that which prevents gizzard erosion was indicated inasmuch as the A & S diet (table 3) gave good growth while producing gizzard erosions of high severity.

Table 2 presents the individual erosion score of each chick as well as the mean erosion scores, their differences and their probable errors. This table illustrates clearly the fallacy of judging by inspection the effectiveness of a particular factor. It will be noted that in the chicks killed at 2 weeks, on the basal diet 40% show slight or no erosion and 60% show marked or severe erosion, while when CSA is added 70% show slight or no erosion and 30% marked or severe erosion. This might seem to indicate that CSA reduced the incidence of erosion, although since two chicks on CSA showed severe erosion as compared with but one on the basal diet it would seem improbable even here that CSA could be a specific factor. However, the difference of the mean scores of the two groups is 0.60 with a probable error of 0.31 which indicates clearly that the scattering within the groups is so great that one cannot draw any conclusion relative to the effectiveness of CSA. This is confirmed again in the chicks killed at 3 weeks, while in those killed at 5 weeks the difference between the means practically disappears and the probable error remains large.

The majority of our observations are condensed in table 3. In the first group (32A), CSA was used to supplement an ordinary commercial chick mash which was adequate for growth and on which it was known that the incidence of gizzard erosion is very small. This experiment was carried out to determine whether CSA would show a growth promoting effect when added to an adequate diet such as it exhibited when added to the Wisconsin diet 454. No effect was observed.

In group 41H the experiment on the Wisconsin diet 454 was repeated, using both commercial and purified CSA. In this group of animals neither commercial nor purified CSA improved growth. The chicks receiving the supplement, in fact, gained less weight than those on the basal ration although the difference was not significant. When commercial CSA

TABLE 3

Tests upon growth promoting and erosion preventing activity of commercial and purified chondroitin sulfuric acid with commercial chick mash, two Wisconsin, and A & S gizzard erosion producing diets

TEST AND DIET	AGE OF CHICKS	BASAL DIET				BASAL DIET PLUS 3% CSA				STATISTICAL ANALYSIS			
		Number of chicks		Mean weight	Mean erosion score	Number of chicks		Mean weight	Mean erosion score	Difference of mean weights	P.E. of difference	Difference of erosion score	P.E. of difference
		Start	End			Start	End						
	weeks												
32A	2	40	35	107		40	40	105		2	± 2.2		
Chick mash	4	40	35	274		40	39	268		6	± 6.5		
	6	40	34	417	1	40	37	413	1	4	±12.4		
41H	2	16	16	93		17	17	89		4	± 2.8		
Wisconsin 454	4	10	10	209	1.2	17	16	188	1.6	21	± 7.8	0.4	±0.12
	2					16[1]	16	95		2	± 3.1		
	4					11[1]	10	192	1.3	17	± 7.1	0.1	±0.10
41K	2	19	19	79		18[1]	18	83		4	± 2.7		
New Wisconsin	4	19	19	146	1.2	18[1]	18	152	1.2	6	± 7.0	0.0	±0.08
41M													
A & S no	2	9	9	119		10	10	132		3	± 7.8		
vitamin K	4	9	2	218		10	7	290					
41N	2	10	10	123		10	10	120		3	± 8.8		
A & S plus	4	10	10	217	1.5	10	10	216	1.2	1	±13.9	0.3	±0.13
vitamin K	2					10[1]	10	119		4	± 7.5		
	4					10[1]	10	216	1.1	1	±13.5	0.4	±0.12
421													
A & S no	2	10	10	132		10	10	132		0	± 6.1		
vitamin K	4	10	5	249	3.8	10	6	274	3.7	25	±14.0	0.1	±0.71
42K													
A & S plus	2	10	10	125		10	10	127		2	± 4.9		
vitamin K	4	10	9	266	2.3	10	10	253	1.7	13	± 7.3	0.6	±0.18
42M	2	10	10	79		10	10	88		9	± 2.7		
New Wisconsin	4	10	10	116	1.9	10	10	126	1.5	10	± 5.2	0.4	±0.19
42-O	2	10	10	105		10	10	101		4	± 4.5		
Wisconsin 454	4	10	10	162	1.6	10	10	163	1.7	1	±10.0	0.1	±0.21

[1] Purified chondroitin sulfuric acid used.

was used with the basal diet, the erosion score was higher and the difference is on the borderline of significance, but with purified CSA the difference disappears. In group 41K, chicks of the same age, weight, and source placed on the new Wisconsin diet and given a supplement of purified CSA failed to show any effect of CSA on growth or erosion.

In group 41M, the A & S diet was used without being supplemented with vitamin K. At 2 weeks the weight differences were not significant; at 4 weeks seven of the nine receiving the basal ration and three of ten receiving the basal ration plus 3% commercial CSA had died of the hemorrhagic diathesis induced, and the numbers are obviously too few for analysis. It may be said, however, that all the chicks remaining as well as those that died showed marked or severe erosion.

The A & S diet was supplemented with a vitamin K extract for group 41N and the mortality reduced to zero. No significant weight differences appear, and although the erosion score of the animals given CSA is lower than that of the chicks on the basal ration, the significance of the difference is questionable.

In groups 42I, 42K, 42M and 42-O, the experiments on the A & S diet with and without vitamin K and on the new and old Wisconsin diets were then repeated with a different lot of chicks. These experiments fully confirm those of the preceding groups. There are no significant weight differences, but CSA has a possibly significant effect upon gizzard erosion in group 42K (A & S diet plus vitamin K). It may be noted that enough chicks survived on the A & S diet in the absence of vitamin K to permit analysis in this case, and that their gizzard erosion score is much higher than that produced by any other ration, although it is not influenced by CSA.

DISCUSSION

Our results may be summarized by saying that in experiments upon 397 chicks divided into eleven groups in which the effect of CSA as a supplement was statistically analyzed,

CSA had a growth-promoting effect in one instance in chicks given the Wisconsin diet 454 and failed to promote growth in four other instances on either the old or new Wisconsin diets. The growth-promoting effect occurred in a group that showed a greater incidence of gizzard erosion than we were later able to obtain upon these diets but there was no significant effect upon gizzard erosion. It seems likely that some constituent of Wisconsin diet 454 varies in its nutritional value. CSA had a possibly significant growth-promoting effect in one instance (group 41M) when the A & S diet was given without additional vitamin K, but failed to promote growth significantly when this diet was given to another group and also failed to promote growth in two instances when the A & S diet was given with vitamin K. It may be noted that all chicks on the A & S diet without vitamin K showed the expected hemorrhagic lesions.

The effect of CSA on gizzard erosions was not indubitably significant in any group. In group 41H (Wisconsin diet 454), it appeared to increase the incidence of erosions, the difference in score being 0.4 ± 0.12; in group 41N (A & S diet plus vitamin K) the difference in erosion score favored CSA being again 0.4 ± 0.12 and in a second group on the A & S diet plus vitamin K CSA may have decreased erosion, the score difference being 0.6 ± 0.18. No differences that suggested a CSA effect appeared in any other group.

Our observations force the conclusion that CSA is not the gizzard erosion preventing factor. The fact that in some instances CSA seems to have a growth-promoting effect and also seems to decrease the incidence of gizzard erosion slightly in some groups may permit us to suspect that this substance has a favorable effect upon the nutrition of the chick in the presence of certain dietary deficiencies. Further studies upon the addition of CSA to deficient diets are now in progress which support this suggestion. If a substance is to be regarded as a specific factor for the prevention of a given deficiency syndrome, it must give unequivocal results. Any possible effect of CSA upon gizzard erosion can only be regarded, in

the light of our analysis, as secondary to a general improvement in nutrition. That CSA has a systemic effect has been shown by previous experiments of Crandall, Roberts and Gibbs ('32) and Crandall, Roberts and Snorf ('36) upon Eck fistula dogs and animals receiving cinchophen. That it is a dietary factor of importance for the chick and the rat will be reported in a separate communication.

An observation incidental to the main objective of this study concerns the differences between the unsupplemented A & S diet and the Wisconsin group diets in the production of gizzard erosion. In our studies it was found that the former diet permits substantially normal growth with a high incidence of gizzard erosions as compared with those of the Wisconsin investigators in which growth is greatly depressed and the incidence of gizzard erosion often relatively low. It should also be pointed out that the addition of vitamin K to the A & S diet produced a marked decrease in the incidence of gizzard erosions, and since the vitamin K was added as a hexane extract supports the contention of these investigators that the erosion preventing factor is ether soluble. We do not, however, offer this as a final conclusion since it seems to us entirely possible that the presence of a hemorrhagic diathesis as in vitamin K deficiency may increase the extent of the gizzard lesions even though there is no change in the erosion preventing factor in the diet. In other words, it is conceivable that vitamin K may decrease the extent of gizzard erosion merely by preventing hemorrhage.

An interesting condition which occurred almost universally in chicks on the Wisconsin diets was a distension and darkening of the cecum. In general this seemed to be somewhat less in the birds receiving CSA, although the difference did not appear in every group. Again one is inclined to consider a non-specific improvement in nutrition rather than a specific effect of CSA upon this condition of megacecum.

SUMMARY

Studies upon nearly 400 chicks given various gizzard erosion producing diets fail to reveal any unequivocal protective action of commercial or purified chondroitin sulfuric acid. This substance does appear, under certain conditions, to have a favorable effect upon growth. It is suggested that any slight influence upon gizzard erosion may be the result of a general nutritional improvement rather than the action of a specifically protective substance. Incidental observations upon chicks receiving gizzard erosion producing diets are discussed.

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SECONDARY DEFICIENCY OF VITAMIN B_1 AND RIBOFLAVIN IN THE BLACKTONGUE PRODUCING DIET [1]

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FOUR FIGURES

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In a previous study dealing with the optimum and minimum dose of nicotinic acid, Margolis, Margolis and Smith ('38) observed that dogs with experimental blacktongue failed to make satisfactory weight gains with treatment after the first and second consecutive attacks. The present study was designed to determine whether the gradual failure of nicotinic acid was due to an accumulated toxic effect or occurred as a result of secondary limiting factors in the diet.

METHOD

Experimental canine blacktongue was produced in adult dogs of mixed breed and both sexes, with an average weight of 16 kg., by feeding them Smith, Persons and Harvey's ('37) modification of Goldberger's diet no. 123 (table 1).

After the development of blacktongue, nicotinic acid was given daily for 10 days and then withdrawn. By this method, standardized by Harvey, Smith, Persons and Burns ('38), one can determine the immediate response to nicotinic acid, as well as the ability to store the active principle. The nicotinic acid, in a concentration of 5 mg. per milliliter, was dissolved

[1] Part of the expenses of this work was covered by grants from the Rockefeller Foundation and the Lederle Biological Laboratories.

in water for oral treatment and in normal physiological saline (0.85%) for intramuscular and subcutaneous injections. Daily doses of 0.5 mg. to 10 mg. per kilogram of body weight were given, each dog receiving the same dosage throughout the 10-day period. The previous results of Margolis, Margolis and Smith ('38) indicated that all doses within this range produced the optimal curative effects for the first attack. The supplements of thiamin (Merck) and riboflavin (Merck and S. M. A.) were given orally.

TABLE 1

Composition of blacktongue-producing diet used

COMPONENTS	QUANTITY APPROXIMATE PER CENT	TOTAL GRAMS [1]	PROTEIN GRAMS	FAT GRAMS	CARBOHYDRATE GRAMS	VIT. B_1 [4] I.U.
Cornmeal, yellow [2]	65	320.0	26.9	15.0	236.8	15–180
Cow peas (Vigna sinensis)	8	40.0	8.5	0.6	24.3	131
Casein,[3] purified	10	48.0	41.6			
Sucrose	5	25.5			25.5	
Cottonseed oil	5	24.0		24.0		
Cod liver oil	5	24.0		24.0		
Salt mixture [5]	2	9.5				
Total	100	491.0	77.0	63.6	286.6	146
Calories			308.0	572.4	1146.4	

[1] Amounts fed daily per dog. Total calories 2027.

[2] Cornmeal, cow peas (coarsely ground) and salt mixture are stirred into water and cooked in an aluminum steam cooker for 1½ hours. Then the other ingredients are poured in and mixed well.

[3] Commercial casein was leached with acidulated water and extracted with 95% alcohol. Details given by Harvey, Smith, Persons and Burns ('38).

[4] Vitamin B_1 requirement for the 16 kg. dog according to Cowgill's formula is 38.61 I.U.

[5] Underhill and Mendel ('28).

RESULTS

A study of nicotinic acid treatment of forty-five attacks of blacktongue in fourteen dogs revealed a decreasing ability of the animals to regain weight after consecutive attacks (table 2, fig. 1). There was also an apparent decrease in the ability of the dog to store the protective factor. It should be emphasized that the condition of the mouth was not so severe before

the third and fourth treatments as it was before the first and second. Treatment became imperative at this point because of the precipitous weight loss.

These results were unexpected since a liver extract [2] was effective in producing repeated satisfactory remissions in

TABLE 2

A total of fourteen dogs on the blacktongue-producing diet treated with nicotinic acid in forty-five repeated attacks (one to four attacks per dog)

CONSECUTIVE ATTACKS	NUMBER OF ATTACKS	AVERAGE WEIGHT GAIN AFTER TREATMENT WITH NICOTINIC ACID	AVERAGE TIME OF RELAPSE AFTER NICOTINIC ACID WAS DISCONTINUED
			days
1st	14	1.58	28
2nd	14	1.40	20
3rd	10	0.76	13
4th	7	0.55	5

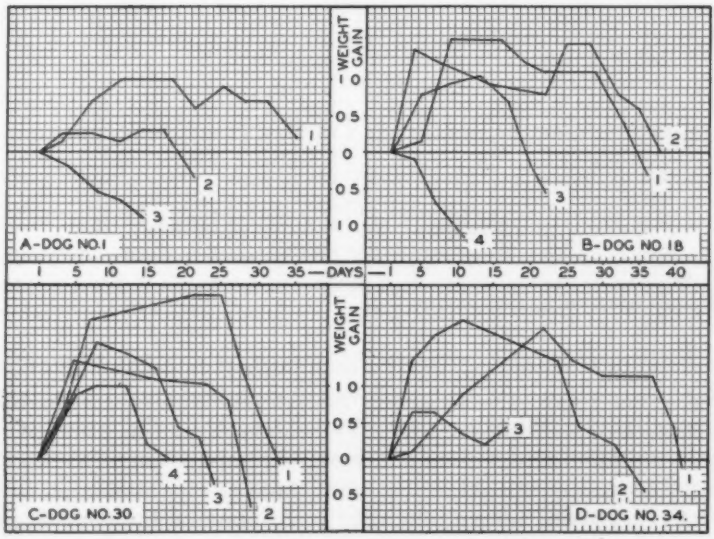

Fig. 1 (A, B, C, D) Weight responses in four dogs on consecutive treatments with nicotinic acid. (The order of treatments is indicated numerically.)

[2] Oral residue and intramuscular extract. Kindly supplied by Lederle Laboratories, Pearl River, New York. This extract was shown by Ruffin and Smith ('37) to be curative of pellagra.

many consecutive attacks of blacktongue. Eleven dogs in sixty-eight attacks of blacktongue showed no decrease of weight response or storage capacity with liver treatment (table 3). Two dogs, after complete failure to respond to nicotinic acid, recovered promptly when given liver and were maintained on liver extract for 3 and 4 months, respectively. After withdrawal of the liver, both dogs relapsed with classical symptoms of blacktongue. One of these dogs then gave an excellent response to nicotinic acid (fig. 3 a), but the other received inadequate amounts and died.

TABLE 3

A total of eleven dogs on the blacktongue-producing diet treated with liver extract in a total of sixty-eight repeated attacks (one to ten attacks per dog)

CONSECUTIVE ATTACKS	NUMBER OF ATTACKS [1]	AVERAGE WEIGHT GAIN AFTER TREATMENT WITH LIVER EXTRACT	AVERAGE TIME OF RELAPSE AFTER LIVER WAS DISCONTINUED
		kg.	*days*
1st	11	1.41	23
2nd	11	1.61	24
3rd	11	1.75	21
4th	9	2.19	20
5th	9	1.49	22
6th	7	2.31	19
7th	4	1.78	14
8th	3	1.77	18
9th	2	1.43	25
10th	1	2.10	

[1] These dogs were in a series reported previously from this laboratory by Harvey, Smith, Persons and Burns ('38).

In view of these findings it was important to determine what factors present in liver in addition to nicotinic acid were responsible for these results. To achieve this, the pure substances associated with the vitamin G complex were fed separately as supplements to the basal diet.

1. Vitamin G (B_2) (riboflavin). In none of the four dogs treated with riboflavin was there any noticeable weight gain or increase in appetite. However, if riboflavin was omitted from the diet entirely or fed in insufficient amounts, there was collapse and finally death, often with total absence of mouth

symptoms. The liver of the one dog examined at necropsy was found to be yellow and to contain large amounts of fat as revealed by section. Both of these findings confirm previous work of Sebrell et al. ('37, '38).

Two dogs, failing with nicotinic acid treatment, were given a supplement of riboflavin alone. The weight decrease continued and there was no change in appetite. Two other dogs, one of whom received no riboflavin and the other only 2 mg. as a supplement during the experimental period of 145 days, responded in their fourth attack of blacktongue with entirely

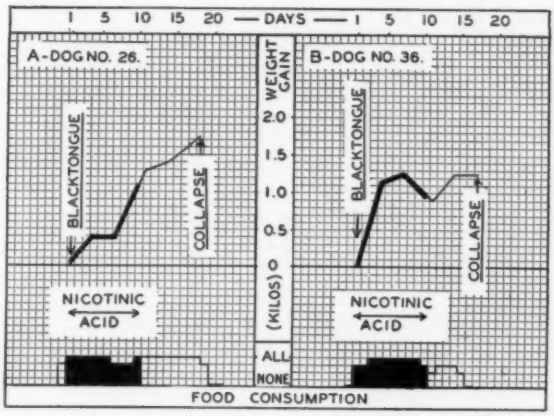

Fig. 2 (A, B) Weight and appetite responses to treatment with nicotinic acid of two dogs with blacktongue complicated by a riboflavin deficiency.

adequate gains in weight and appetite when nicotinic acid was given, but died in collapse 7 and 10 days after the drug was discontinued. In both cases collapse occurred at the peak of the weight curve (fig. 2, a, b). One of these dogs had received adequate amounts of thiamin.

In the riboflavin deficient dogs we also observed a very characteristic type of dermatitis involving loss of hair and a scaly condition of the skin. This condition seemed to parallel the degree of riboflavin deficiency, yielding gradually to treatment with riboflavin. The condition is distinguished from

mange by failing to heal with mange cure. Goldberger and Wheeler ('28) noted changes in the skin of the scrotum of their dogs with blacktongue, and Sebrell and Onstott ('38) noted a dermatitis quite similar to the type reported here in their riboflavin deficient dogs. Adams ('36) and Smith ('38) have also observed skin changes in vitamin G deficiency.

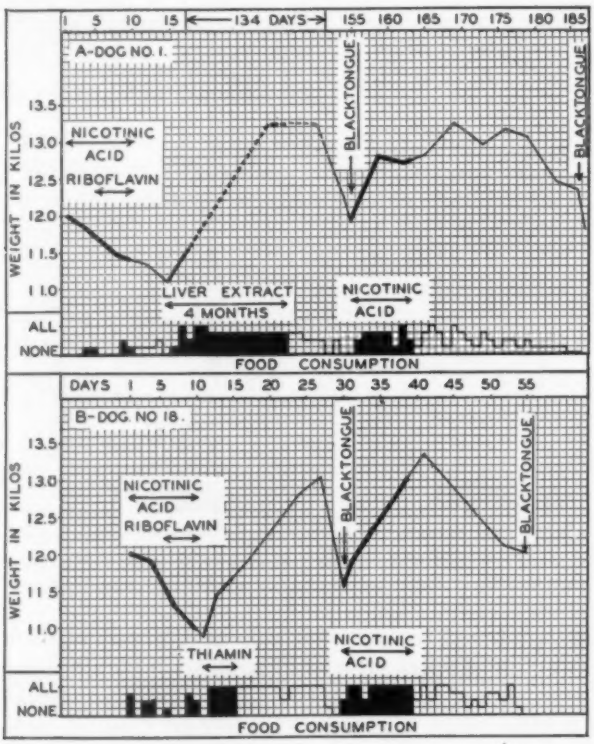

Fig. 3 (A). Weight response with nicotinic acid treatment of the third attack of blacktongue (fig. 1 a, curve 3), continued weight loss with addition of riboflavin, restoration with liver extract and subsequent weight response to nicotinic acid. (B). Weight response with nicotinic acid treatment of the fourth attack of blacktongue (fig. 1 b, curve 4), continued weight loss with addition of riboflavin, restoration with vitamin B_1 (thiamin), and subsequent weight response to nicotinic acid.

2. Vitamin B_1 (thiamin chloride, Merck). No neurological symptoms of vitamin B_1 deficiency were observed during these experiments. It was possible to maintain one dog in excellent health for 11 months on the basal diet supplemented by nicotinic acid and riboflavin. But after one or two successive attacks of blacktongue, dogs began to show loss of appetite, inadequate weight gains, and finally falling weight curves in the absence of mouth symptoms. These symptoms were not altered by the administration of nicotinic acid or riboflavin. Four dogs were treated with vitamin B_1 (thiamin) with the following results: One dog responded with a 2.15 kg. gain in weight after complete failure to respond to nicotinic acid; two dogs, after showing a decreased response to nicotinic acid, improved with B_1, gaining 1.95 and 1.85 kg. respectively. A fourth dog which was still responding well to nicotinic acid had, at the end of the customary depletion period, a mouth that was only slightly suspicious of blacktongue [3] but had loss of weight and appetite. When 15 mg. of B_1 was fed, the weight and appetite increased promptly while the mouth lesions were advancing to a picture of severe blacktongue (fig. 4 a). The increased demand for vitamin B_1 with repeated attacks of blacktongue had no relation to the amount or severity of diarrhea in these dogs.

3. Adenylic acid (from muscle). Adenylic acid, 5 mg. fed daily for 10 days, when added to the diet after failure of nicotinic acid had no perceptible effect on the two dogs tested.

4. Nicotinic acid, riboflavin and thiamin. Seven dogs on the blacktongue producing diet were tested over a period of 7 to 13 months by supplementing the diet with riboflavin, thiamin and nicotinic acid. At the end of the experimental period all of these dogs were in excellent condition, at approximately their original weight, and free from macrocytic anemia (table 4).

Three of these dogs were maintained for 11 to 12 months by continuous administration of daily doses of 1 mg. thiamin

[3] We have found along with other investigators that this mild condition of slightly reddened buccal mucosa has a tendency to come and go

and 1 mg. of nicotinic acid per kilogram of body weight and weekly doses of 1 to 2 mg. of riboflavin. At the end of this period there was no evidence of cumulative toxic effect from the nicotinic acid.

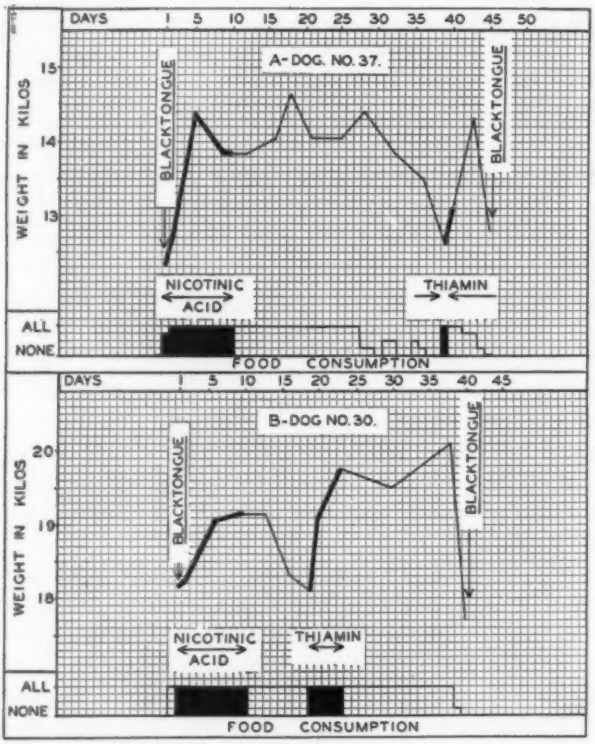

Fig. 4 (A). Anorexia and weight loss in the absence of blacktongue after second treatment with nicotinic acid, and subsequent weight and appetite response to vitamin B_1 (thiamin), while the mouth symptoms progressed to severe blacktongue. (B). Weight response in fourth treatment with nicotinic acid (fig. 1 c, curve 4), and subsequent weight gain with vitamin B_1 (thiamin). This curve demonstrates adequate storage of nicotinic acid in vitamin B_1 deficiency.

In figures 2, 3 and 4 food consumption is indicated by the four squares at the bottom of the chart, each square representing one-fourth of the daily ration. During treatment periods the food consumption is indicated in black. This period is also indicated by arrows on chart and by the heavier line in the weight curve.

Four other dogs were maintained on the basal diet supplemented at intervals with riboflavin and thiamin in amounts sufficient to prevent deficiency from these factors. Under these conditions twenty attacks of blacktongue, identical in

TABLE 4

Dogs maintained on the blacktongue-producing diet supplemented with riboflavin, thiamin and nicotinic acid

DOG NO.	PERIOD OF TREATMENT	HGB.	R.B.C.
Dogs given B_1 and riboflavin and nicotinic acid regularly			
	months	*gm.*	
30	12	14.9	6,005,000
32	11	14.2	6,130,000
33	11	9.2	4,900,000
34 [1]	11	16.2	7,300,000
Dogs given B_1 and riboflavin regularly, nicotinic acid only in attacks of blacktongue			
18	13	15.4	7,500,000
37	9	13.0	5,445,000
49	7	12.6	5,300,000
51	7	13.0	5,500,000

[1] This dog did not receive vitamin B_1 (thiamin).

TABLE 5

A total of four dogs on the blacktongue-producing diet supplemented with thiamin and riboflavin treated with nicotinic acid in twenty repeated attacks (one to six attacks per dog)

CONSECUTIVE ATTACKS	NUMBER OF ATTACKS	AVERAGE WEIGHT GAIN AFTER TREATMENT WITH NICOTINIC ACID	AVERAGE TIME OF RELAPSE AFTER NICOTINIC ACID WAS DISCONTINUED
		kg.	*days*
1st	4	2.09	29
2nd	4	1.63	24
3rd	4	2.20	29
4th	4	2.06	20
5th	2	2.25	25
6th	2	2.58	

every respect with the first attack on the unsupplemented diet, developed at regular intervals. The standard nicotinic acid treatment of these attacks gave uniformly satisfactory results comparable to those obtained with liver (table 5). One of

these dogs, after failing to respond to repeated treatments with nicotinic acid (fig. 1 c) while on the unsupplemented diet, gave satisfactory results with nicotinic acid in six consecutive attacks of blacktongue after the diet was supplemented with riboflavin and thiamin.

DISCUSSION

It is evident from the data submitted that the blacktongue producing diet used in these experiments is deficient in riboflavin. This deficiency has been demonstrated in the rat by Booher and Hansmann ('36) and Helmer and Fouts ('38) and in the dog by Zimmerman et al. ('34), Cowgill et al. ('37) and Sebrell et al. ('37 a, b, '38). We were able to confirm the finding of these investigators that in the absence of riboflavin, dogs die in collapse. It should be emphasized, however, that although the blacktongue diet is deficient in riboflavin, a factor undoubtedly required by the dog, it is not significant in the etiology of blacktongue. It has no curative effect on acute blacktongue, while on the other hand, dogs responding to nicotinic acid with prompt cure of the symptoms, often die suddenly in collapse due to riboflavin deficiency (fig. 2).

Helmer and Fouts ('38) observed that a powdered liver extract completely supplements the blacktongue producing diet when rats serve as the experimental animal. The present study shows that for the dog, liver extract also completely supplements the blacktongue producing diet, and further, that the combination of nicotinic acid, riboflavin and vitamin B_1 will replace the liver.

Although it was possible for us to maintain one dog in excellent condition for 11 months on the basal diet plus nicotinic acid and riboflavin, a vitamin B_1 deficiency was regularly produced in dogs suffering repeated attacks of blacktongue (figs. 1, 3 and 4). These attacks, so like pellagra in their periodic recurrences, gastro-intestinal lesions, sore mouth, infection and metabolic disturbance, obviously led to an increased demand of the dog for vitamin B_1. This greater re-

quirement could not be correlated with food consumption [4] or diarrhea [5] which was never of sufficient duration or severity in our experiments to play an important part.

In the presence of vitamin B_1 deficiency the specific effect of nicotinic acid on the mouth symptoms was not altered nor was the storage period of this factor decreased. The apparent interference with storage which is indicated in table 2 was due to complete nutritional failure which necessitated treatment before the mouth symptoms were severe. At this early stage of the experiment no other treatment than nicotinic acid was considered. It was subsequently found that for normal response to nicotinic acid with prompt gain in weight and appetite an adequate supply of vitamin B_1 is essential.

Since the natural blacktongue producing diet, on which the experimental ones are based, and the natural pellagra producing diet as found in the endemic areas of the South are almost identical it is not surprising to find peripheral neuritis occasionally associated with pellagra (Sydenstricker and Armstrong, '37). The present study indicates that while vitamin B_1 is deficient in the diet and required for the well being of the dog it is not etiologically significant in the production of blacktongue, the analogue of human pellagra.

In patients with endemic pellagra peripheral neuritis has never been considered part of the syndrome, whereas it is a significant and diagnostic part of the beri beri syndrome (Eddy and Dalldorf, '37). It is well known that vitamin deficiencies in the human are complicated and rarely, if ever, limited to one factor. Where peripheral neuritis occurs in association with pellagra, the neuritic symptoms may be cured by vitamin B_1, while the pellagra symptoms grow worse (Spies and Aring, '38; Smith and Ruffin, '38).

[4] According to Cowgill's formula the daily requirement of vitamin B_1 for the 16 kg. dog is 38.61 I.U. The portion of the diet fed supplies in the uncooked state 146 to 311 I.U. of the vitamin (Fixsen and Roscoe, '38). The exact amount of destruction of vitamin B_1 by cooking the natural carriers (cornmeal and cow peas) in the presence of the salt mixture is not known.

[5] Dann and Cowgill ('38) found that the vitamin B_1 requirement for the dog was increased by an artificially induced diarrhea.

It is to be expected, then, that vitamin B factors with their various combinations in natural foodstuffs will give rise to deficiencies characterized by different combinations of symptoms. The primary or predominant deficiency determines the character of the syndrome which results, although there may be various accompanying symptoms attributable to other minor deficiencies.

SUMMARY AND CONCLUSIONS

Thirty-three dogs in 133 attacks of blacktongue were studied in their response to treatment with 1) liver, 2) nicotinic acid alone, 3) adenylic acid from muscle, and 4) nicotinic acid combined with vitamin B_1 (thiamin) and vitamin B_2 (riboflavin).

1. A study of sixty-eight attacks of blacktongue in eleven dogs indicated that liver extract completely supplements the blacktongue producing diet under the conditions of our experiments.

2. A study of forty-five attacks of blacktongue in fourteen dogs revealed the inability of nicotinic acid to replace liver as a complete supplement to the diet, although in the first and often in the second attack the results with nicotinic acid were comparable to those with liver.

3. Two dogs were treated with adenylic acid but results were not conclusive.

4. Twenty attacks of blacktongue in four dogs maintained on the basal diet supplemented with vitamin B_1 (thiamin) and vitamin B_2 (riboflavin) when treated with nicotinic acid gave results that were in every way comparable with the results obtained with liver. Three dogs were maintained on the basal diet supplemented with these three factors for 11 to 12 months in normal condition. There was no cumulative toxic effect from the nicotinic acid in these three dogs, nor was there any macrocytic anemia in any of the seven dogs.

It is concluded then that under the conditions of these experiments both riboflavin and vitamin B_1 (thiamin) are secondary deficiencies of our blacktongue producing diet. It appears also that the dog's requirement for vitamin B_1 is markedly increased by repeated attacks of blacktongue.

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ON THE DIGESTIBILITY OF RAW POTATO STARCH IN MAN

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In 1920 the previously debated question as to whether raw starch is utilizable by man was apparently settled in the affirmative by the observations of Langworthy and Deuel ('20). They found that relatively large quantities (426 to 773 gm.) of raw corn or wheat starch could be fed to normal human subjects without any appearing in the feces. Raw potato starch proved to be less 'digestible' than the other two, but, even so, they were able to recover in the feces only 5 to 38% of that (468 to 710 gm.) ingested. These observations appear to be largely responsible for the fact that it is now sometimes stated in textbooks that large quantities of raw starch can be digested and assimilated by man.

During the course of various in vitro experiments, we were forced to the conclusion that raw potato starch is almost entirely refractory to the action of gastro-intestinal enzymes. Since this was apparently inconsistent with the observations of Langworthy and Deuel ('20), we have repeated and extended their study on the digestion of raw potato starch by normal human subjects.

METHODS

In vitro experiments. Pancreatin was used in conducting the in vitro experiments since it offered a reproducible source of highly active enzymes. The preparation employed throughout the study possessed the following activity: trypsin, double

U. S. P. standard; lipase, as determined by the method described by Cherry and Crandall ('32), 4400 units per gram; diastase, as determined by Willstater's method (Schmidt, Greengard and Ivy, '34), 7 units per gram.

The quantity of reducing substance produced by incubating raw potato starch with an excess of pancreatin was compared with that produced by comparable treatment of the same quantity of boiled starch. Each determination was controlled by determining the reducing substances formed from the pancreatin alone and from the raw starch alone. Thus, by considering the quantity of reducing substance produced by the digestion of the boiled starch as representing total possible digestion, it was possible to calculate the percentage digestion of the raw starch. The proceduce in detail was as follows.

Four 125-cc. Erlenmeyer flasks were prepared as is shown in the following tabulation:

Flask no.	*Quantity of starch (mg.)*	*Treatment of starch*	*Quantity of pancreatin (cc. 2% sol.)*	*Quantity of buffer (pH 6.9) (cc.)*
1	500	Raw	5	25
2	500	Boiled	5	25
3	500	Raw	0	30
4	0	.....	5	25

The flasks were incubated at 37°C., with sufficient aggitation to keep the starch in suspension, for periods ranging from 3 to 20 hours. At the end of the incubation period the material was filtered and the reducing sugar concentration of the filtrates was determined by the hypoiodate titration method (Schmidt, Greengard and Ivy, '34). The total quantity of reducing substance found in flasks 3 and 4 was subtracted from that found in flasks 1 and 2 respectively, leaving the quantity of reducing substance produced by the enzymatic digestion of the starch.

In vivo experiments: ten healthy medical students were used as subjects for the experiment. The composition of the starch diet, which was essentially the same as that used by Langworthy and Deuel ('20), was milk 63%, raw potato starch 21%, peanut oil 8.7% and sucrose 7.3%.

During the first test period on five of the subjects the diet was prepared in the form of a frozen pudding, thus reduplicating the experimental conditions of Langworthy and Deuel. During subsequent tests the diet was consumed as an unfrozen fluid. In all cases a readjustment period of 1 month was permitted between consecutive tests on any given subject.

The tests were conducted in the following manner. The subjects were given 1281 gm. of the starch diet per day for a 3-day period (nine meals). Thus, during each 3-day period a total of 807 gm. of starch was ingested. During this period they were permitted to drink tea, coffee and fruit juices as desired, but no other food. A carmine 'marker' was given with the first meal of the period and again with the last. All feces were collected between the first appearance of the first dose of carmine and the complete disappearance of the second dose. The starch content of the feces was determined by a method which we have previously described (Beazell, Schmidt and Ivy, '37).

The first phase of the study was concerned with the determination of the effect of freezing on the digestibility of the raw starch. Five of the subjects were given the diet in the form of a frozen pudding and the fraction of the ingested starch lost in the feces was determined. Subsequently the test was repeated except that the diet was unfrozen. In the second phase of the study the other five subjects were employed to determine the effect of large doses of a potent diastatic enzyme on the utilization of the raw starch. The starch loss was determined first, during a control period, and second, during a period in which 8 gm. of 'takadiastase' were taken with each meal. 'Takadiastase' was chosen for the study because it is a potent source of diastatic enzyme, and because it is inactivated at a relatively low pH. This latter quality permits the major portion of that ingested to survive the gastric acidity (Ivy, Schmidt and Beazell, '36).

RESULTS

In vitro experiments. The results of a number of these experiments are shown in table 1. It will be seen that the quantity of reducing sugar obtained from the digestion of the raw starch never exceeded 7.6% of that obtained from the digestion of the boiled starch. Furthermore, lengthening the incubation period to 20 hours did not increase the quantity of raw starch digested. This latter fact suggests that a constant fraction of the raw starch may have been more easily digested than the remainder, perhaps as a result of mechanical rupture of the envelope of the starch granule during milling.

TABLE 1

In vitro digestion of potato starch

(500 mg. starch incubated with 100 mg. pancreatin)

TRIAL	INCUBATION PERIOD	REDUCING SUGAR (NET)[1]		PER CENT DIGESTION OF RAW STARCH
		From boiled starch	From raw starch	
	hours			
1	3½	367	28.0	7.6
2	5	418	19.8	4.7
3	20	500	23.0	4.6

[1] Expressed as milligrams maltose.

In vivo experiments. The effect of freezing the starch on the quantity lost in the feces is clearly demonstrated by the results recorded in table 2. During the period when the subjects ingested the starch meal in the form of a frozen pudding, the average starch loss of the five subjects was 26%. Thus, freezing caused less starch to be lost in the feces.

The addition of the 'takadiastase' to the diet had no effect on the quantity of starch lost in the feces. The results of this phase of the study are recorded in table 3. It will be seen that the differences between the two periods are all attributable to spontaneous variations.

Flatulence usually accompanied by cramp-like pain was a prominent and distressing symptom in all of the subjects. In some cases it was sufficiently severe (++++ flatulence) to

be incapacitating. Since it is obviously impractical to make quantitative gas collections in a study of this nature, it was necessary to evaluate the degree of flatulence on the bases of the subjective sensations of the subjects. Thus, any attempt

TABLE 2

Effect of freezing on the quantity of ingested raw starch excreted in the feces (values are for entire metabolic period)

SUBJECT	STARCH DIET FROZEN					STARCH DIET UNFROZEN				
	Wet weight feces	Dry weight feces	Total starch in feces	Per cent ingested starch excreted	Flatulence	Wet weight feces	Dry weight feces	Total starch in feces	Per cent ingested starch excreted	Flatulence
	gm.	gm.	gm.			gm.	gm.	gm.		
J.B.	933	311	195.4	24.2	++++	1439	417	310	38.2	+++
J.J.	944	262	191.6	23.6	+++	1514	486	342	42.2	+
R.M.	1203	400	317.8	39.2	+++	1575	512	426	53.0	++
H.G.	562	225	195.4	24.2	++	1383	458	344	42.5	+
R.B.	635	160	149.4	18.5	++++	953	292	227	28.0	+++
Av.	855	271	209.9	26.0		1373	433	329.8	40.9	

TABLE 3

Effect of orally administered diastase on the quantity of ingested raw starch excreted in the feces (values are for entire metabolic period)

SUBJECT	CONTROL PERIOD (DIET UNFROZEN)					ENZYME PERIOD (DIET UNFROZEN)				
	Wet weight feces	Dry weight feces	Total starch in feces	Per cent ingested starch excreted	Flatulence	Wet weight feces	Dry weight feces	Total starch in feces	Per cent ingested starch excreted	Flatulence
	gm.	gm.	gm.			gm.	gm.	gm.		
R.R.	1356	309	130.6	15.4	++++	1723	420	172.8	21.4	+++
R.C.	1306	522	363.0	41.6	+++	1457	456	289.0	35.6	+++
J.S.	1462	573	415.4	49.0	++	1606	596	446.0	55.0	+++
W.V.	1851	662	462.7	57.3	+++	2047	719	461.3	57.2	+++
W.B.	1670	480	290.4	36.0	+	1563	466	283.0	35.0	+++
Av.	1529	509	330.4	39.7		1679	527	330.4	40.8	

to draw conclusions from minor changes in the reported degree of flatulence would be hazardous. However, the first group of subjects uniformly reported that they were much less flatulent while ingesting the unfrozen meal than they had

been while ingesting the frozen meal. In every case the change was so marked that there was little room for questioning its significance.

DISCUSSION

Langworthy and Deuel ('20) conducted a series of seven experiments on five subjects in their investigation of the digestibility of raw potato starch. They employed the frozen pudding in all of their tests. The average starch loss in their series was 21.8% (4.8 to 37.7%). The slight differences between this value and that obtained by us, when the frozen pudding was employed (26.0%) can be attributed to individual variation.

The results of the first phase of the in vivo experiments establishes beyond any reasonable doubt that freezing does modify the quantity of starch lost in the feces. In the case of each of the five subjects the quantity lost was significantly greater during the period in which the unfrozen meal was being ingested. The differences are all too marked to be attributed to spontaneous individual variations. Justification for this latter statement can be found in the uniformity of results between the two periods in the subsequent experiment in which the unfrozen diet was ingested during both periods (table 3).

It should be noted that throughout this paper the term 'starch loss' has been employed rather than 'starch utilization' or 'starch digestion.' The latter two terms imply useful digestion; the term 'starch loss' is noncommittal. When all of the facts are correlated, it appears evident that bacterial fermentation accounts for a significant fraction of the raw starch that disappears during passage through the human gastro-intestinal tract. In the first place, the uniform extreme flatulence establishes active fermentation, and the fact that flatulence was greatest when the starch loss was least (frozen pudding period) suggests that fermentation played an important part in the increased break down of the starch.

In the second place, the failure of large doses of potent diastase to affect the starch lost in the feces, indicates that amylolytic enzymes are of minimal importance. Finally, it is difficult to reconcile the results of the in vivo and in vitro experiments unless bacterial fermentation is taken into account.

CONCLUSIONS

1. The significance of values for the 'digestibility' of raw starch is open to question when they have been obtained by feeding the starch in the form of a frozen pudding.

2. The fact that a given quantity of raw starch disappears during passage through the gastro-intestinal tract does not mean that all that has disappeared has been usefully digested and assimilated. Indeed, the evidence suggests that in the case of raw potato starch, bacterial fermentation accounts for an important part of the starch that is decomposed during passage through the alimentary tract.

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DIET AND RATE OF DEPLETION OF HEPATIC VITAMIN A [1,2]

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INTRODUCTION

It is generally recognized that the liver is the chief depot for vitamin A storage. Baumann, Riising and Steenbock ('34) showed that when these stores were drawn upon, the rate of depletion was directly proportional to the amount of vitamin A present. Davies and Moore ('35) and Ruiz ('35) reported that very large stores of vitamin A were rapidly depleted until a certain level was reached; after that the rate of depletion was much slower. Greaves and Schmidt ('36) found that choledochocolonostomized rats or rats given thyroxine were depleted more rapidly than normal rats. Thyroidectomized rats showed a decreased rate of depletion, while icteric, laparotomized and dinitrophenol-treated rats depleted their reserves at rates comparable to normal rats. The relationship of vitamin A and thyroxine has recently been reviewed by Murlin ('38). Green ('34) noted that young rats survived for approximately the same length of time on vitamin A-free diets which differed greatly in fat content.

In connection with out earlier studies (Lease, Lease, Weber and Steenbock, '38) on the effect of rancidity on vitamin A

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destruction, the question arose as to whether rancid fats tended to destroy hepatic stores of vitamin A. It has been shown by several workers that rancid fats in a diet containing vitamin A or carotene tend to induce a vitamin A deficiency (Fridericia, '24; Powick, '25; Mattill, '27; Nelson, Nelson and Lowe, '34; Lease, Lease, Weber and Steenbock, '38). However, the effect of rancid fat on liver stores of vitamin A had not been investigated. The experiments now reported deal with the effect of high-, low- and rancid-fat rations on this aspect of the problem. The effect of speedy depletion of excess fat from the liver by the use of choline (Best and Huntsman, '35) on vitamin A reserves was also investigated.

METHODS

The rats were fed a basal ration composed of casein 18%, brewers' yeast 8%, salts (Osborne and Mendel, as modified by Hawk and Oser, '31) 5%, and sucrose 69%. When fat was included, it was added at the expense of an equivalent amount of sucrose. The fresh lard was a high quality, kettle-rendered product. The rancid lard was prepared by aerating the fresh lard with moist air at 50°C. in the presence of 1 mg. of copper acetate per 100 gm. of fat and with intense illumination from Mazda lamps. Halibut liver oil [3] was used as the source of vitamin A. The choline hydrochloride was incorporated in the ration after being dissolved in a small amount of water.

The vitamin A content of the livers was determined colorimetrically (Lease, Lease, Weber and Steenbock, '38), using the Carr and Price ('26) antimony trichloride reagent. The fat content of the livers was determined by titration of the liberated fatty acids of the fat as outlined by Hynd and Rotter ('30).

RESULTS AND DISCUSSION

Effect of rancid fat. Uniform reserves of vitamin A were produced in rats 4 weeks of age by giving 1500 blue units of

[3] The halibut liver oil was the commercial Haliver Oil furnished by Abbott Laboratories.

vitamin A as halibut liver oil daily for 4 days. Two days later their livers were found to contain an average of 1048 blue units. These reserves were allowed to deplete on diets low in vitamin A but high, respectively, in fresh or rancid fat. After various periods of time the vitamin A content of the livers of a few animals from each group was determined. Analysis after 14, 28 and 56 days revealed no significant difference in the stores of rats which had been fed 30% of rancid lard or 30% of fresh lard (table 1). The rancid lard used in these experiments was only moderately rancid, having a peroxide number of 30.

TABLE 1

Effect of fresh and rancid fat rations on hepatic stores of vitamin A [1]

WEEKS ON DIET	BLUE UNITS OF VITAMIN A PER LIVER		
	Initial	30% fresh lard	30% rancid lard per. no. 30
Initial	1048		
After 2 weeks		845	902
After 4 weeks		555	556
After 8 weeks		482	450

[1] Each value represents an average of four or more rats analyzed individually.

In another experiment, the effect of very rancid fat was determined in older animals. Adult males of similar weight, age and dietary history as taken from the stock colony were given fresh lard or very rancid lard (peroxide number 200) for a period of 40 days. To facilitate consumption of the ration its fat content was increased approximately 10% weekly, starting at a 30% level and ending with 67%. Analyses of the livers revealed similar rates of depletion irrespective of whether fresh or rancid fat rations had been fed.

Effect of high- and low-fat diets. In some of our experiments the fat content of the diets was high enough to produce fatty livers during the depletion period. It was possible that the amount of fat in the diet had some effect on vitamin A stores and their metabolism. The effect of dietary fat on the depletion of vitamin A previously stored was, therefore, investigated.

For this purpose rats 4 weeks of age were placed on the basal ration and given 1 drop of halibut liver oil (1500 blue units of vitamin A daily) for 5 days. Three days later an average of 2114 blue units of vitamin A per liver was found. Half of the animals were continued on the basal ration containing no additional fat; the other half were given the 30% fresh lard ration. Analyses of livers at intervals for 15 weeks (table 2), showed that the animals on the high-fat diet lost slightly more vitamin A than those on the low-fat ration. However, there was no clear-cut distinction between the two. In a second feeding trial extending over 8 weeks, entirely similar results were obtained.

TABLE 2

Effect of high and low fat rations on the rate of vitamin A depletion [1]

WEEKS ON DIET	BLUE UNITS VITAMIN A PER LIVER		
	Initial	Basal ration + 30% fresh lard	Basal ration
Initial	2114		
3 weeks		1974	2086
6 weeks		1344	1722
9 weeks		1162	1236
12 weeks		601	671
15 weeks		334	411

[1] Each value represents an average of three or more rats analyzed individually.

From these data it appears that the rate of depletion of moderately high reserves of vitamin A is not greatly affected by the amount of fat in the diet. These results are in line with those of Green ('34) who concluded that the more obvious steps of fat metabolism do not involve the use of vitamin A.

Influence of fat mobilization. Since vitamin A and its esters are fat-soluble, it seemed possible that the rapid depletion of fat from a fatty liver might increase the rate of unloading its vitamin A. To obtain information on this point, rats 3 weeks of age were given the basal ration containing 30% of fresh lard for a period of 40 days. On the first, fourteenth and twenty-eighth day of feeding they were given graded amounts of vitamin A as shown in table 3. On the fortieth

day a few representative animals were killed for analysis. The remaining animals were divided into two groups; one was continued on the basal ration, while the other was given a supplement of 1.5% of choline. Analyses of the livers of representative animals revealed the rapid loss of fat on the choline diet, and none from the other; the vitamin A reserves were the same for both (table 3).

After the major part of our work had been completed, Thorbjarnson and Drummond ('38) reported that the rate of disappearance of vitamin A from the liver was greater with

TABLE 3

Effect of choline on the rate of depletion of vitamin A from fatty livers[1]

DAYS ON DIET	TOTAL BLUE UNITS OF VITAMIN A FED	ON BASAL RATION		ON BASAL RATION + 1.5% CHOLINE	
		Blue units vitamin A per liver	Per cent of fat in liver	Blue units vitamin A per liver	Per cent of fat in liver
0	3000	616	16.6		
4		612	16.3	598	9.8
12		585	16.6	564	1.5
20		545	17.0	539	1.7
0	4500	1072	14.6		
12		963	14.1	1111	5.7
0	9000	2409	13.6		
12		2035	15.9	2233	6.6

[1] Each figure represents an average of four or more livers analyzed individually.

a low-fat diet than with a high-fat diet and that choline plus the high-fat diet tended to increase the rate of depletion. They used rats with very large stores of vitamin A. Our data are related to more physiological levels of storage. Thorbjarnson and Drummond were working with animals with enormous stores of vitamin A induced by feeding approximately 9000 blue units per day for 4 weeks. As Davies and Moore ('35) have shown, considerable variation exists in the amount of vitamin A contained in the livers of rats fed massive doses. They also showed that when liver stores are very high, very rapid depletion takes place over a short period of time, after which a much slower and more steady depletion is observed. Thorbjarnson and Drummond were studying the influence of fat and choline at this period of rapid and

variable depletion, which may well account for the divergence in results.

SUMMARY

The rate of loss of vitamin A from the liver of the rat was not affected greatly, if at all, by the amount of fat in the diet, by the feeding of rancid fats, nor by the rapid depletion of fat from the liver as effected by the administration of choline.

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THE BIOLOGICAL VALUE OF CAROTENE IN VARIOUS FATS[1,2]

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INTRODUCTION

In the early days of vitamin A research, little attention was given to the nature of the fat in which the vitamin was administered. However, variations were soon observed in the growth response of rats when carotene or vitamin A was given in different solvents. Failure to demonstrate the vitamin A activity of carotene in ethyl oleate (Duliere, Morton and Drummond, '29) was attributed to the instability of carotene in this solvent (Hume and Smedley-MacLean, '30; Drummond, Ahmad and Morton, '30). Marked differences in the stability of vitamin A in various solvents were found by Dann ('34), and similar variations in the stability of carotene were demonstrated by Baumann and Steenbock ('33). The stability of carotene in ethyl oleate or ethyl laurate was found to be markedly increased by the use of antioxidants (Olcovich and Mattill, '31). When hydroquinone was added to carotene in an 'unstable solvent,' the carotene became stable, and vitamin A activity could be demonstrated. It appeared that the variations in biological results obtained with different solvents were due to the various degrees of instability of the active materials in these solvents.

[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station, Madison.

[2] The authors wish to express their appreciation to Lever Brothers Company whose financial support made this work possible.

More recently, however, it has been claimed that certain variations in biological response could not be attributed to the destruction of the active agent before administration. The evidence is reviewed in a report of the Medical Research Council ('35). Lathbury and Greenwood ('34) reported that different samples of arachis oil and coconut oil varied in their suitability as solvents for assaying vitamin A or carotene biologically and that quinol did not render an inferior oil suitable. Dyer, Key and Coward ('34) reported that "a solution of the standard (carotene) in arachis oil had five to six times the potency of solutions in hardened cottonseed oil and ethyl laurate, though made of the same strength . . . . Cod liver oil Z dissolved in coconut oil (161441) was about three times as active as when dissolved in hardened cottonseed oil or olive oil." De ('37) reported that large doses of carotene appeared to be better utilized when fed in red palm oil than in foods such as amaranth. Kraybill and Shrewsbury ('36) studying the growth response of rats fed carotene in cottonseed oil [3] and in decolorized butterfat observed that in order to obtain a given growth response, two to three times as much carotene was needed in treated butterfat as in cottonseed oil. They reported that carotene was stable in their samples of treated butterfat. Coward ('36) made comparisons of the variation in response of rats fed carotene dissolved in various oils, and concluded that there was no difference in the extent of the variations.

Variations in the potency of carotene dissolved in various oils have also been attributed to differences in absorption from the tract. The deleterious effect of mineral oil on carotene absorption is well known (Dutcher et al., '34; Jackson, '31, '34). In view of the large number of determinations of vitamin A and carotene made on foodstuffs by chemical methods, it is of practical importance to determine to what extent such analytical results are a true measure of biological potency. Obviously, if the potency of a milligram of carotene varies with the medium in which it is administered, chemical

[3] Wesson oil brand.

data can have only limited biological application. Kraybill and Shrewsbury ('36) have already intimated that chemical analyses are inadequate as a measure of the vitamin potency of butterfat. We have, therefore, studied the biological response of rats to carotene administered in various oils, using growth, cure of ophthalmia and the storage of vitamin A in the liver as biological criteria.

EXPERIMENTAL

Vitamin A storage. The effect of various fats on the storage of vitamin A in the liver was studied as follows: rats 3 to 4 weeks of age were placed on a ration consisting of casein 18, yeast 8, salts (Osborne and Mendel as modified by Hawk and Oser, '31) 4, dextrin 40, fat 30. The fats studied were decolorized butterfat, lard, soy bean oil, cottonseed oil,[4] and hardened cottonseed oil.[5]

The butterfat used had been decolorized as follows (Kraybill and Shrewsbury, '36): 20 gm. of Lloyd's reagent were added to 800 gm. of butterfat in a 2-liter flask at 50°C. and the mixture stirred periodically for 8 hours under nitrogen. The fat was filtered with suction through a wad of cotton on quantitative filter paper. The treatment with Lloyd's reagent and filtration was repeated. The product was practically free from color and proved to be without vitamin A activity, since it failed to restore growth and cure ophthalmia when fed to six depleted rats at a level of 30% in a vitamin A-free diet.

For the tests, the carotene or vitamin A was dissolved in the fats so that 1 gm. of ration contained 12 micrograms of carotene or 12 blue units of vitamin A as halibut liver oil.[6] Most of these rations were kept at 0°C. in the dark, in completely filled glass containers, and were prepared fresh every 4 days. However, in view of the instability of carotene in decolorized butterfat, rations containing such butterfat were kept at a temperature of —8°C.

[4] See footnote 3, page 92.

[5] Covo brand, made by hydrogenating cottonseed oil to an iodine number of approximately 70.0. The saponification number was 196.0.

[6] The halibut liver oil was a commercial brand known as Haliver Oil furnished by the Abbott Laboratories.

Six rats were placed on each of the diets. They were kept in separate cages and were fed equivalent amounts of food; 5 gm. daily the first week; 6 the second, 7 the third, and 8 gm. daily thereafter. This amount of food was consumed shortly after being given, thus minimizing the possibility of destruction in the food cup. At the end of 5 weeks the livers were analyzed for vitamin A by the $SbCl_3$ technic (Carr and Price, '26) described previously (Lease, Lease, Weber and Steenbock, '38), and the results expressed as blue units (Moore, '30).

Marked increases in the liver stores of vitamin A resulted from the administration of either carotene or vitamin A, regardless of the kind of fat in the basal diet (table 1). Initial

TABLE 1

Liver stores of vitamin A produced by carotene fed in various fats

TYPE OF FRESH FAT	AVERAGE BLUE UNITS OF VITAMIN A PER LIVER [1]	
	Fed 12 micrograms of carotene per gram of ration	Fed 12 blue units of vitamin A per gram of ration
Lard (Swift's Premium)	566	508
Covo	548	579
Soy bean oil	542	640
Wesson oil	502	530
Butterfat (free of vitamin A and coloring matter)	574	537

[1] Each figure is an average of results from six animals.

stores averaged 50 to 75 blue units of vitamin A per liver; final stores, 540 blue units (502 to 640). The character of the fat failed to influence the storage of vitamin A at these relatively high levels of intake.

Growth response to carotene. Considerable carotene was excreted when sufficient amounts were fed to produce hepatic stores of vitamin A. Hence a modifying effect of the fat may have been masked by the presence of excess carotene. The possible influence of various fats on carotene utilization was, therefore, studied at low levels of carotene intake, using the growth response and the cure of ophthalmia of depleted rats as criteria.

Carotene dissolved in decolorized butterfat was stable for 2 months when stored in vacuo at —8°C., but was markedly unstable in decolorized butterfat. When stored in small corked flasks from which the air had been partly displaced by bubbling nitrogen through the solution, 80% of the carotene was destroyed within a week at 0°C. In contrast with this, carotene was completely stable in most other oils under similar storage conditions. The degree of instability in decolorized butterfat varied with the severity of the decolorizing treatment. Thus, carotene was relatively stable in butterfat treated with amounts of Lloyd's reagent just sufficient to remove the color in 20 minutes at 45°C. Such a preparation, however, was not suitable as a medium for assaying carotene biologically because it still contained enough vitamin A to cure ophthalmia and promote growth when fed at a 20% level.

For the growth experiments, rats 35 to 50 gm. in weight were fed our vitamin A-low basal ration (heated casein 18, modified Osborne and Mendel salts 4, hydrogenated vegetable fat 10, brewers' yeast 7.9, irradiated yeast 0.1, and sucrose 60) until symptoms of ophthalmia appeared and growth ceased. They were then transferred to a similar low-A ration containing only 3% of fat, the fat being the same as that in which the supplement was administered, namely, cottonseed oil or decolorized butterfat. Carotene was fed daily as a separate supplement dissolved in the oils so that 1 to 2 drops contained 1 microgram of carotene. One or 2 micrograms of carotene were fed daily. The supplements were fed for 8 weeks.

Since the object of the study was a search for factors other than stability which might influence the biological efficiency of carotene, a rigid check was kept on the carotene content of the oils used. This was particularly necessary for the decolorized butterfat solutions because their carotene content was steadily decreasing. Fresh solutions were made up every 5 to 6 days and the carotene content determined spectrophotometrically (Baumann and Steenbock, '33) every third day. The number of drops fed per week was adjusted according to the results of the analyses, so that 7 or 14 micrograms of carotene were consumed per rat per week.

The more stable solutions, such as carotene in cottonseed oil, were prepared every second week, and stored under nitrogen at —8°C. in the dark. Aliquots were removed to a smaller flask twice a week. The carotene content of the solutions was determined spectrophotometrically at weekly intervals.

In spite of the marked difference in the stability of carotene in cottonseed oil as compared with decolorized butterfat, an identical growth response resulted from the administration of equal amounts of carotene in the two fats (table 2). This identity was observed at both the 1 and 2 microgram levels of intake. There was evidently no need to postulate a difference in the biological efficiency of carotene in these two media, when adequate precautions were taken to ensure an equality of intake.

The growth response of depleted rats to daily supplements of 1 microgram of carotene in various other oils was determined for cottonseed oil,[7] lard, coconut oil, mineral oil, soy bean oil, linseed oil, triolein, and two samples of peanut oil. One of the peanut oils was a 'refined' oil obtained commercially and the other a crude oil prepared in the laboratory by cold-pressing raw peanuts. In addition, 2 micrograms of carotene in refined peanut oil were fed daily, and 5 micrograms daily in cottonseed oil and in mineral oil. The triolein was prepared by heating molecular proportions of glycerol and oleic acid by the method of Garner ('28). The oils were fresh and of good quality. The peroxide numbers ranged from 0.08 for the decolorized butterfat to 0.94 for the lard. The free fatty acid content varied from 0.04% (expressed as oleic acid) for the cottonseed oil to 0.94% for the decolorized butterfat.

Bertram ('37) reported the presence of small amounts of carotene in several oils including linseed and soy bean oil. Our samples of these oils were also found to contain some carotene. Concentrates prepared from these oils revealed vitamin A activity. These findings may explain the reported

[7] See footnote 3, page 92.

TABLE 2

The relative vitamin A potency of carotene fed in Wesson oil and decolorized butterfat

SUPPLEMENT	AVERAGE GAIN IN WEIGHT AT END OF						
	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1 microgram carotene per day, decolorized butterfat (4 rats)[1]	12.8 (8–19.5)	24.8 (17–34)	35.5 (25–47)	45.7 (31–65)	52.2 (43–63)	58.1 (47–69)	66.1 (56–75)
1 microgram carotene per day, Wesson oil (6 rats)	2.5 (−9–12)	21.0 (11–32)	33.4 (27–42)	45.7 (35–58)	58.0 (51–71)	62.7 (53–82)	68.7 (62–92)
2 micrograms carotene per day, decolorized butterfat (6 rats)	8.3 (3– −13)	25.0 (21–30)	39.2 (31–50)	47.6 (32–62)	58.7 (44–81)	68.1 (42–103)	78.0 (51–119)
2 micrograms carotene per day, Wesson oil (6 rats)	13.0 (10–18)	27.0 (21–32)	45.6 (34–58)	56.0 (44–68)	63.3 (51–84)	73.7 (57–92)	80.0 (62–99)

[1] Two animals from this group were discarded because of respiratory trouble.

biological superiority of linseed oil as a solvent for carotene (Basu, '37). We accordingly did not use these oils in the basal ration, and applied a correction factor for pigments when they were used as solvents for feeding carotene in small amounts.

Considerable differences in growth were obtained when equal amounts of carotene were fed in the various oils (table 3). Essentially the same amount of growth, which was quite substantial, was obtained with coconut oil, cottonseed oil, lard,

TABLE 3

The growth response of rats to carotene fed in various media

MEDIA	NUMBER OF RATS	CAROTENE GIVEN DAILY	AVERAGE GAIN AT END OF					RANGE AT END OF 5 WEEKS
			1 week	2 weeks	3 weeks	4 weeks	5 weeks	
		micrograms	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Cottonseed oil	14	1	4	22	37	44	63	45 to 83
Coconut oil	10	1	3	19	35	47	55	40 to 83
Lard	10	1	10	25	41	53	63	40 to 94
Peanut oil (cold pressed)	6	1	11	32	50	67	79	59 to 105
'Refined' peanut oil (commercial)	11	1	7	13	22	32	36	3 to 70
Soy bean oil	3	1	11	31	46	61	70	50 to 82
Linseed oil	4	1	5	20	29	35	40	29 to 56
Triolein	10	1	7	19	27	35	40	29 to 47
Refined peanut oil (commercial)	4	2	7	27	48	58	60	49 to 70
Cottonseed oil	4	5	19	39	57	70	79	57 to 101
Mineral oil	4	5	−8	−11	−15	−17	−37	−18 to −55

soy bean oil and cold-pressed peanut oil, 55 to 79 gm. in 5 weeks, but growth on the other oils was definitely inferior.

The inferiority of the 'refined' peanut oil was observed in two experiments carried out at two different times. It was even more pronounced than the growth averages indicated, since these values represented only the averages for those animals which survived the 5-week period. Four other animals had died without recovering from the symptoms of ophthalmia. Many developed severe respiratory infections,

notwithstanding the fact that the animals were actually in better physical condition than those on the other oils when the supplement was first administered. Only an occasional animal from the other groups died during the experimental period, except in the mineral oil group which never recovered from the effects of the vitamin A deficiency, even though 5 micrograms of carotene were fed daily. This latter is in line with the well-known experience of others and merits no further discussion.

The poor growth observed with the supplements of carotene in 'refined' peanut oil, triolein and linseed oil may have been due to a number of factors. There was no evidence that it was due to poor absorption. When rats were given 20 mg. of carotene daily in 'refined' peanut oil in comparison with cottonseed oil, no difference in the carotene excretion was observed. Furthermore when the stability of carotene in the different fats was determined at temperatures of 0, 20 and 37°C. greater stability was observed with peanut oil than with coconut oil (table 4) although growth was better on the latter. As a matter of fact too much emphasis should not be given to the weight increments as an index of carotene or vitamin A values because they may well have been determined by organoleptic or other properties of the oil inimical to physiological well-being, with the further possibility that their harmful effect was accentuated by the low level of vitamin A metabolism. Use was accordingly made of the degree and the rate of cure of ophthalmia as a more specific index. Practically all of the rats had incipient ophthalmias when the carotene solutions were given but occasionally responsiveness to carotene was delayed by respiratory infections. Data on animals thus infected were generally discarded.

The data on the cure of ophthalmia indicated that the relatively poor growth on 'refined' peanut oil, linseed oil and triolein was not due to a deficiency of vitamin A. Cures on these fats were obtained on the average in 15 to 27 days. On lard, soy bean oil, Wesson oil and coconut oil, as examples of the oils more efficient for growth, cures were obtained in

TABLE 4

Stability of carotene in various fats

SOLVENT	PER. NO.	FREE FATTY ACIDS	INITIAL CONCENTRATION	PER CENT CAROTENE REMAINING											
				1 week			2 weeks			1 month			2 months		
				0°	20°	37°	0°	20°	37°	0°	20°	37°	0°	20°	37°
		%	*micro-grams/cc.*												
Butterfat	0.05	0.19	108		90	100	94	88	88	94	94	89	96	89	66
Treated [1] butterfat	0.08	0.94	98.5	14	13	8	5	8	3	Tr	Tr	Tr	0	0	0
Cottonseed oil	0.15	0.04	84	96	100	100	97	100	84	97	92	83	97	83	68
Treated [1] cottonseed oil	0.12	0.04	109	94	94	85	87	92	76	95	95	77	92	83	66
Fresh lard	0.94	0.12	91	76	81	88	83	69	69	87	73	42	82	52	25
Coconut oil	0.54	0.05	92.5	84	91	69	84	88	11	93	56	0	69	10	0
'Refined' peanut oil (commercial)	0.65	0.12	93.5	84	81	68	90	72	45	89	72	45	76	67	19

[1] Treated with Lloyd's reagent.

about the same time, viz., 12 to 23 days. Furthermore at least one failure to effect a complete cure of ophthalmia in 30 days resulted with each of the fats with the exception of the cold-pressed peanut oil with which complete cures were obtained in 12 days. As rats on this fat were started promptly on the carotene supplement in anticipation of a poor response, their average was not included with the others. We do not feel justified in crediting this crude peanut oil with superior attributes except insofar as it was superior to the 'refined' oil in the promotion of growth.

It was also interesting to note that treatment of cottonseed oil with Lloyd's reagent had no effect on the stability of carotene although such treatment induced properties in butterfat which were later destructive.

SUMMARY

We did not succeed in confirming the work of others that decolorized butterfat gives growth promoting properties to carotene inferior to those obtained with cottonseed oil. When excessive amounts of carotene or vitamin A were fed in lard, soy bean oil, cottonseed oil, devitaminized butterfat and a hydrogenated vegetable fat,[8] no marked differences in storage were obtained. Approximately equal growth responses were obtained when 1 microgram of carotene daily was fed in cottonseed oil, soy bean oil, lard, decolorized butterfat, coconut oil or crude peanut oil. Inferior growth was obtained on triolein, linseed oil and 'refined' peanut oil, but the rate of cure of ophthalmia was essentially the same on all oils.

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